The Koala and its Retroviruses:
Implications for Sustainability and Survival

edited by

Geoffrey W. Pye, Rebecca N. Johnson, and Alex D. Greenwood

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Preface

to Papers Presented at the Koala Retrovirus Workshop, San Diego Zoo, April 2013

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This volume presents peer-reviewed papers from the oral presentations and break-out group-sessions delivered at the *San Diego Zoo Global Koala Conservation Workshop: The Koala and its Retroviruses: Implications for Sustainability and Survival* meeting, held at San Diego Zoo, 17–18 April 2013. Over 70 participants from Australia, Europe, Japan, and North America attended, including experts in the fields of koala care, conservation, ecology, epidemiology, immunology, molecular biology, population management, retrovirology, veterinary medicine, and zoonoses.

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The recognition of lymphoid neoplasia in koalas (Backhouse & Billinger, 1960; Canfield *et al*., 1987) and its likely association with a retrovirus (Canfield *et al*., 1988; Worley *et al*., 1993; Hanger *et al*., 2000; Tarlinton *et al*., 2005) stimulated research amongst virologists as they excitedly studied what they believed to be the first real-time endogenization of a retrovirus (Tarlinton *et al*., 2006; Stoye, 2006; Oliveira *et al*., 2007; Tarlinton *et al*., 2008). More recent work has shown a suspected exogenous spread of KoRV in southern Australia (Simmons *et al*., 2012) as well as an extension of the possible time line of the endogenization (Ávila-Arcos *et al*., 2013).

In addition, research has demonstrated the possibility of trans-species transmission (Fiebig *et al*., 2006), identification of the KoRV receptor as PiT1 (Oliveira *et al*., 2006), identification of the virus in koalas in Japanese and German zoos (Fiebig *et al*., 2006; Miyazawa *et al*., 2011) and the ability to detect presence of the virus in fecal material (Miyazawa *et al*., 2011).

The recent isolation of a variant from the originally sequenced koala retrovirus, isolated from koalas dying from lymphoid malignancies in a North American zoo (Xu *et al*., 2011; Xu *et al*., 2013), and the concern it generated about population management prompted San Diego Zoo Global

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to identify the need for a workshop to initiate the expansion of KoRV knowledge from foundational research to applied research, in order to promote the sustainability and survival of the koala.

The first day of the workshop was based on the current state of foundational research of KoRV, the level of impact of KoRV on both captive and free-ranging koala populations, the different disease entities that may be related to KoRV infection, koala immunology, and what future research is needed to further our understanding of KoRV. The second day was based on the need for applied research, what we could extrapolate from other well-researched retrovirus models (e.g., HIV treatment, FeLV vaccination), the role of KoRV in Chlamydial infections in koalas, strategies to reduce the spread and disease expression of KoRV, and to determine the zoonotic risk of KoRV.

The research presented and discussed at the two-day workshop demonstrated that although much progress has been made in understanding KoRV and its influence on koala health, a great deal remains to be learned and further empirical scientific data gathered to improve our understanding of this retrovirus in koalas.

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Xu, W., C. K. Stadler, K. Gorman, N. Jensen, D. Kim, H. Zheng, S. Tang, W. M. Switzer, G. W. Pye, and M. V. Eiden. 2013. An exogenous retrovirus isolated from koalas with malignant neoplasias in a US zoo. *Proceedings of the National Academy of Sciences, USA* 110(28): 11547–11552.
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A Novel Exogenous Retrovirus Isolated from Koalas *(Phascolarctos cinereus)* with Malignant Neoplasias in a United States Zoo

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Abstract. Koalas in US zoos were screened for koala retroviruses in an effort to determine the viral mechanism for koala retrovirus induced malignant neoplasias. Although the previously characterized koala retrovirus (KoRV-A) was present in all US koalas, some koalas were also infected by a novel koala retrovirus, termed KoRV-B. The genome of KoRV-B is highly related to KoRV-A; however, certain regions within the viral genome, including the envelope gene, displayed diversity. These differences are sufficient to allow KoRV-B to employ a receptor (a thiamine transporter) that differs from that used by KoRV-A (a phosphate transporter). Of great interest was the strong correlation between the presence of KoRV-B and malignant disease (lymphomas) in koalas. All koalas that died from lymphoma were KoRV-B positive as were the dead joeys ejected from the pouch of KoRV-B positive dams. We found no evidence of KoRV-B transmission from sires to offspring but did from dam to offspring through de novo infection, rather than via genetic inheritance like KoRV-A. Detection of KoRV-B in native Australian koalas should provide a history, and a mode for remediation, of leukemia/lymphoma currently endemic in this population.

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Endogenous retroviruses (ERVs) have played an integral role in mammalian evolution. Elements derived from these genetically inherited ERVs comprise as much as 8% of the human genome (Bromham, 2002) and are known to regulate the expression of highly conserved gene clusters (van de Lagemaat *et al*., 2003). The majority of ERVs are defective remnants of exogenously transmitted retroviruses that likely integrated into the germline of mammalian progenitors millions of years ago. The discovery of koala retrovirus (KoRV) (Hanger *et al*., 2000) described the first endogenous retrovirus that is still actively producing infectious particles capable of transspecies transmission while being retained as an inherited part of the host genome. KoRV isolates described to date in Australia, Germany, and Japan have shown very limited genetic diversity (>99% sequence identity), characteristic of an endogenous virus. However, considering the likelihood that koala genomes also contain newly integrated forms of KoRV, we screened cohorts of 13 koalas from the Los Angeles Zoo (LAZ) and 28 koalas from the San Diego Zoo (SDZ) to detect more diverse KoRV isolates (Xu *et al*., 2013).

PCR amplification of viral sequences from koala specimens obtained from the LAZ was performed using genomic DNA prepared from blood or tissue and from viral RNA present in plasma, with primers specific to KoRV. Additionally, a viral marker rescue assay was developed using human cells containing an integrated replication incompetent retroviral genome that expresses GFP (green fluorescent protein). The GFP genome can be rescued and assembled into virus if KoRV is present in the koala peripheral blood mononuclear cells (PBMCs) co-cultured with the human-GFP cells. If KoRV rescues the GFP genome then supernatant containing KoRV-GFP vectors can infect naïve target cells that will

subsequently express GFP.

PCR of infected target cells using primers for the KoRV *env* gene and the long terminal repeats (LTR) confirmed the existence in all assessed koalas from both SDZ and LAZ of a KoRV envelope gene almost identical to the endogenous KoRV previously described. Notably, a heretofore-uncharacterized KoRV envelope gene sequence was also identified in blood or tissue samples from six of 13 koalas from the LAZ, including three koalas that died of lymphoid leukemias and a joey ejected from the pouch of an infected dam at approximately one month of age. We refer to this new KoRV isolate as KoRV subgroup B or KoRV-B, and the original isolate as KoRV-A in keeping with the nomenclature previously established for other gammaretroviruses. Detection of KoRV-B envelope sequences was independently confirmed at the Centers for Disease Control (CDC) lab using freshly collected blood taken at multiple time points from the same koala.

We obtained the complete genome of KoRV-B from PBMC-derived genomic DNA using primers specific for the novel KoRV-B envelope gene sequences and primers derived from viral sequences flanking and within the LTR. KoRV-B differs from KoRV-A in the U3 region of the LTR (the region containing the viral promoter, and transcription regulatory sequences) and in its envelope gene. The U3 regions are represented at both ends of the integrated retroviral genome and can also direct expression of host genes flanking the viral integration site. If the adjacent gene is an oncogene, viral promoter activation of that gene can promote cancer. The envelope of KoRV-B differs significantly from KoRV-A in the receptor-binding domain (RBD). KoRV-B also contains the amino acid residue motif CETTG in its RBD. This motif is present in the RBD of all envelope proteins of infectious gammaretroviruses except for KoRV-A isolates and non-inducible ERVs (Oliveira *et al*., 2007).

KoRV-A and KoRV-B viruses exhibit different host ranges in cell culture, which indicates that they may use different receptors to infect cells. Murine MDTF cells are resistant to KoRV-A and KoRV-B, however expressing the human ortholog of the KoRV-A receptor confers susceptibility to infection by KoRV-A. The normal cell function of the KoRV-A receptor is that of a phosphate transporter (SLC20A1, formerly reported in the literature as PiT1). PiT1 has been reported to function as the viral receptor for gibbon ape leukemia virus (GALV) and feline leukemia virus subgroup B (FeLV-B) (Overbaugh *et al*., 2001). MDTF/PiT1 cells are susceptible to KoRV-A but resistant to KoRV-B, a finding consistent with KoRV-B using a receptor different from that used by KoRV-A to infect susceptible cells. Because gammaretroviruses tend to employ transporters as receptors, we individually expressed a panel of transporters in MDTF cells to determine whether any of these tested transporters conferred susceptibility to KoRV-B. Using this approach we discovered KoRV-B infects via the thiamine transporter (formerly referred to as THTR1 and now recognized as SLC19A2). The thiamine transporter was previously shown to be the receptor for feline leukemia virus subgroup A (FeLV-A) (Mendoza *et al*., 2006).

KoRV-B does not appear to be vertically transferred in the germline. KoRV-B positive sires do not transmit KoRV-B to their offspring if the dam is KoRV-B negative. KoRV-B positive dams can transmit KoRV-B to their offspring when the sire is KoRV-B negative. Necropsy tissue from a KoRV-B positive six-week old joey that died in pouch and was ejected from its KoRV-B positive dam is consistent with KoRV-B being transmitted in utero or in milk ingested in the pouch.

Most KoRV-A isolates from the 38 koalas analyzed from SDZ and LAZ contain envelope sequences closely related to or in many cases identical to the previously reported KoRV-A envelope sequences. However, genetic and phenotypic diversity in KoRV is well represented by KoRV-B, which utilizes thiamine transporter THTR1 (SLC19A2) as a receptor. It is possible that KoRV-B is a recombinant between the endogenizing KoRV-A and existent KoRV sequences in the koala genome, much like the origin of FeLV-B, a recombinant of exogenous FeLV-A and endogenous FeLV-B envelope sequences (Overbaugh *et al*., 2001). Whether KoRV-A serves as a founder virus in a manner analogous to FeLV-A giving rise to different KoRV subgroups/variants in addition to KoRV-B will need further investigation. Sequencing the koala genome will help resolve the composition of endogenous retroviral fragments that may have contributed to the generation of KoRV-B and other KoRV variants.

The correlation between the presence of KoRV-B infectious virus and malignant disease in koalas is strong even though the assessed sample size is small and we cannot exclude participation of KoRV-A in the observed pathology. Nonetheless, the ability to assess KoRV-B status, and therefore the likelihood of susceptibility to neoplastic malignancy could be of tremendous importance in sustaining and managing the koala population in captivity and better understanding the epidemiology of KoRV infection. Preventing KoRV-B-positive dams from breeding, sequestering KoRV-B-positive koalas from the rest of the koala population, and developing a KoRV vaccine may all be sensible approaches to reducing the impact of KoRV-B infection on the koala population.

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The Evolution of Koala Retroviruses: Insights from other Endogenous Retroviruses

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Abstract. The koala retrovirus (KoRV) is associated with outbreaks of *Chlamydia* and leukemia in wild and zoo koalas *(Phascolarctos cinereus)*. Although endogenous retrovirus-like elements (ERVs) are common in the genomes of all vertebrates (comprising ca 8% of the human genome), KoRV is the only retrovirus known to be currently in the process of transitioning from exogenous to endogenous form. Here, we examine how other host-pathogen interactions, including other host-ERV systems, can inform our understanding of KoRV in koalas. We note that as an exogenous retrovirus becomes endogenous, there would be a dramatic reduction in mutation rates, which may shift the process of accommodation from the pathogen to the host. The low genetic diversity present in koalas may be in part responsible for the failure of the species to develop genetic resistance to KoRV. Isolation between koala populations may have hindered the geographic spread of the virus, but may also hinder selective sweeps of beneficial host alleles or beneficial proviral mutations, thereby precluding rapid increases in host fitness. In humans, some ERVs are involved in normal host functions such as placentation, or in the pathogenesis of diseases such as Hodgkin's lymphoma. However, ERVs present in humans and other species are ancient, precluding prospective studies of germ line invasions. By contrast, the ongoing invasion of the koala germ line by KoRV provides a singular opportunity to study retroviral endogenization as it is occurring. This research can benefit the health of both humans and koalas.

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Endogenous retroviruses are common elements present in the genomes of all vertebrates examined, with ca. 8% of the human genome comprised of retrovirus-like elements (Bromham, 2002; Weiss, 2006; Pontius *et al.*, 2007; Blikstad *et al.*, 2008). Although some ERVs play a functional role in host health and disease in humans and other species (Roy-Burman, 1995; Mi *et al.*, 2000; Lamprecht *et al.*, 2010), most ERVs exist as "junk DNA" with highly disrupted coding regions and no functional role (Roca *et al.*, 2004; Roca *et al.*, 2005; Pontius *et al.*, 2007). Comparisons across the genomes of humans and other primates, and of other vertebrate lineages, have shown that ERVs have resulted from multiple invasions of and proliferations in the host germ line by retroviruses (Johnson & Coffin, 1999; Blikstad *et al.*, 2008; Polani *et al.*, 2010). Despite being ubiquitous, almost all known ERVs endogenized many thousands or millions of generations ago, making it difficult to infer the events that occur during and shortly after the invasion of a host germ line by an endogenizing retrovirus (Weiss, 2006; Blikstad *et al.*, 2008).

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The koala retrovirus (KoRV) appears to represent an exceptionally recent invasion of a host germ line by a retrovirus (Hanger *et al.*, 2000; Stoye, 2006; Tarlinton *et al.*, 2006). Unlike any other known ERV, KoRV appears to be present in endogenous form in only some but not all members of the host species (Stoye, 2006; Tarlinton *et al.*, 2006; Simmons *et al.*, 2012). Some populations of koala in southern Australia appear to be free or largely free of KoRV (Stoye, 2006; Tarlinton *et al.*; 2006; Simmons *et al.*, 2012). KoRV also appears to persist as an exogenous virus, and thus provides the opportunity to study the transition of a retrovirus from exogenous to endogenous form on a "real time" basis (Stoye, 2006; Tarlinton *et al.*, 2006; Simmons *et al.*, 2012).

KoRV is associated with pathologies that affect both wild and zoo koalas, most notably *Chlamydia* infection and the formation of leukemias (Canfield *et al.*, 1988; Hanger *et al.*, 2000; Tarlinton *et al.*, 2005; Fiebig *et al.*, 2006; Oliveira *et al.*, 2006; Oliveira *et al.*, 2007). In recent studies, we found that the functional features present today in KoRV have remained largely unchanged for more than a century (Ávila-Arcos *et al.*, 2013). We also found that KoRV was already ubiquitous in northern Australian populations in the late 1800s, suggesting that the spread of KoRV geographically has been limited since at least that time (Ávila-Arcos *et al.*, 2013). Finally, the genetic variability of koalas, previously reported to be low in living populations, was found to be similarly low in ancient museum samples as well (Tsangaras *et al.*, 2012). Here we examine how other host-pathogen systems can inform approaches to KoRV in koalas. We specifically examine other host-ERV interactions and how they can inform our understanding of KoRV, although the examples will include non-ERV and non-retroviral examples when these appear to be relevant.

Host-pathogen accommodation: potential role for population size and mutation rates

The evolution of a host-pathogen system may involve a process of co-adaptation between the pathogen and the host (Kerr, 2012). When a pathogen enters a new species, it may be especially pathogenic to the novel host. However, there may in some cases be evolutionary pressures for a virus to become less pathogenic over time (Kerr, 2012). Host genetic variation that provides resistance to the virus will be selected for, and any host variant that provides protection would be expected to undergo a selective sweep, becoming more common in the population (May & Anderson, 1979).

An important model for host-pathogen interaction is the myxoma virus infection of European rabbits in Australia (Kerr, 2012). Myxoma virus is a poxvirus naturally found in and benign to American rabbits (genus *Sylvilagus*). However, the virus is deadly to European rabbits, which are an invasive species and a major pest in Australia. In 1950, myxoma virus was released into the Australian rabbit population, spreading quickly across the continent. Initially the case fatality rate for infected rabbits was 99.8% (Kerr, 2012). But the virus quickly became attenuated, with a case fatality rate of 90% by the second season, suggesting that there was selective pressure, if only initially (May & Anderson, 1990), for the virus to become less deadly (Kerr, 2012). In time, the host species also became more resistant to the virus. Rabbits exposed to one particular grade of virus went from 90% to 26% fatality over 7 generations, as genetic variants that made rabbits less susceptible to the virus became more common each generation (Kerr, 2012).

In considering the adaptation of myxoma virus and rabbits to each other, it is important to note that adaptations are likely to impact the pathogen population more quickly than they impact the host population (Mulvey *et al.*, 1991; Kerr, 2012). The genetic variation present within a lineage varies with mutation rate and population size (Tajima *et al.*, 1998; Duffy *et al.*, 2008). Each infected rabbit may carry a very large number of copies of the virus, thus the population size of the virus would be greater than that of affected rabbits, and the virus would also have a shorter generation time (Mulvey *et al.*, 1991; Duffy *et al.*, 2008; Kerr, 2012). This in turn would lead to a relatively larger number of new mutations in the virus, which would allow for greater adaptability of the virus to the rabbit than vice versa (Mulvey *et al.*, 1991; Duffy *et al.*, 2008).

This example of host-pathogen co-adaptation may be relevant to the koala-KoRV system. When KoRV first infected koalas as an exogenous retrovirus, the virus rather than the koala may have undergone most of the initial mutation that would drive the host and parasite to accommodate each other (Duffy *et al.*, 2008). This may be especially true given that koalas appear to suffer from reduced genetic diversity (Wilmer *et al.*, 1993; Tsangaras *et al.*, 2012). KoRV appears to have developed a number of protein motifs that reduce its virulence vs. the closely related gibbon ape leukemia virus (GALV) (Oliveira *et al.*, 2006; Oliveira *et al.*, 2007). It may not be surprising that KoRV appears to have evolved this lowered virulence before becoming endogenized (Ávila-Arcos *et al.*, 2013). Invasion of the koala germ line by KoRV may have been difficult before the mitigating mutations, since any endogenous KoRV that killed its host before it reached reproductive age could not have persisted. A greater understanding of why KoRV is currently not deadly enough to prevent sufficient numbers of host offspring from reaching reproductive age may provide insights into how to also protect older koalas.

KoRV would be present in very high copy number in each infected koala, thus having a much higher population size than the koala host (Duffy *et al.*, 2008). Furthermore, exogenous KoRV, with an RNA genome that lacks the genomic repair mechanisms of the host, would have a much higher mutation rate than the koala, which has DNA repair mechanisms that limit the mutation rate (Duffy *et al.*, 2008).

One of the critical recent findings made by our group is that KoRV has changed little in the past century (Ávila-Arcos *et al.*, 2013). This may be due to the reduction in mutation rate that would occur once a retrovirus endogenizes (Duffy *et al.*, 2008). Once endogenized, KoRV becomes subject to cellular DNA-repair mechanisms. Thus the mutation rate for endogenous KoRV is likely to be substantially lower than the rate for exogenous KoRV, slowing the adaptive potential of the retrovirus relative to that of the host, once the virus transitions to endogenous copies.

Adaptation between ERV and host: the evolution of protective ERVs

KoRV is the only ERV for which some individuals of the host species are believed to be completely free of proviral copies (Stoye, 2006; Tarlinton *et al.*, 2006; Tarlinton *et al.*, 2008; Simmons *et al.*, 2012). In other host species ERVs may be insertionally polymorphic, i.e., present at a particular locus in only some individuals (Turner *et al.*, 2001; Roca *et al.*, 2005). Nonetheless, even in these cases, all members of the species will carry ERV copies at other loci (Turner *et al.*, 2001; Roca *et al.*, 2005). In the case of KoRV, many individuals especially in southern populations may be completely free of endogenous proviruses, an indication that the germ line of the koala has only been invaded recently relative to known

ERVs in other species (Stoye, 2006; Tarlinton *et al.*, 2006; Tarlinton *et al.*, 2008; Simmons *et al.*, 2012). Furthermore, KoRV appears to be strongly pathogenic in koalas (Hanger *et al.*, 2000; Tarlinton *et al.*, 2005; Oliveira *et al.*, 2006; Oliveira *et al.*, 2007; Tarlinton *et al.*, 2008), while most ERVs in other species appear to be benign. Since vertical transmission in general tends to select for lower virulence (Toft & Karter, 1990), this may be another indication of a recent origin for KoRV. An examination of how ERVs in other species may have become innocuous may provide insights into the future of KoRV in the koala.

One relevant example may be the endogenous feline leukemia viruses (enFeLVs) present in the germ line of the domestic cat and related species (Polani *et al.*, 2010). The presence of enFeLVs in several closely related species of the genus *Felis* suggests that these ERVs began proliferating in the germ line of an ancestor of domestic and wild cats some 3–6 million years ago (Johnson *et al.*, 2006; Polani *et al.*, 2010). That has been sufficient time for many enFeLVs to develop mutations that disrupt the open reading frames (ORFs) of the provirus, although at least one copy of enFeLV retains its ORF structure, indicative of a relatively recent integration event (Roca *et al.*, 2004; Pontius *et al.*, 2007). Mutations in enFeLV after it endogenized would occur at the slow rate of change that occurs in the genome of the host species (Roca *et al.*, 2004). Yet even this slow rate has been sufficient to disrupt most copies of enFeLV in the domestic cat, rendering enFeLVs non-functional due to frame-shift or other disruptive mutations, or to other mechanisms that can block the proliferation of selfish DNA (Roca *et al.*, 2004; Pontius *et al.*, 2007). The high pathogenicity of KoRV in koalas may suggest that insufficient time has elapsed for a general breakdown of the structure of genomic copies of KoRV, although further studies would be needed to establish this definitively.

Interestingly, some enFeLVs in the cat germ line appear to play a protective role in the host species. It appears that viral transcripts of the *env* gene encoded by a domestic cat enFeLV produce partial envelope protein, which is secreted by cells (McDougall *et al.*, 1994). This partial protein appears to block entry into the cells of exogenous FeLV of strains that share envelope similarity with the endogenous forms (McDougall *et al.*, 1994). Thus, an enFeLV codes for an envelope protein that interferes with infection by similar exogenous viruses (McDougall *et al.*, 1994). Such a protective effect would be expected to lead to positive selection, increasing the frequencies of the protective ERV in host populations. An analogous protective role also appears to have evolved in some mice within the genus *Mus*. In mice, a retroviral restriction gene *Fv1*, has been found to be derived from the *gag* region of an ERV (Best *et al.*, 1996; Yan *et al.*, 2009). This ERV appears to code for a protein product that appears to interact with exogenous murine leukemia viruses, restricting the ability of the exogenous virus to proliferate (Best *et al.*, 1996; Yan *et al.*, 2009).

Koala biology and protective host genetic variants against KoRV

While ERVs may develop a protective role within the host, there is also evidence that some host genetic variants will provide protection against retroviruses. Protective allelic variants in the host species would be expected to increase over time due to selective pressure by the pathogen against individuals that lack protection (May & Anderson, 1979). Host genes with allelic variants that mediate responses to retroviruses have been well studied in the case of human immunodeficiency virus (HIV-1) (O'Brien & Nelson, 2004; An & Winkler, 2010; Zhao *et al.*, 2012). Several dozen human genes have been found to have allelic variants that are beneficial (or detrimental) to humans exposed to HIV-1 (O'Brien & Nelson, 2004; An & Winkler, 2010; Zhao *et al.*, 2012). For example, HIV-1 uses the transmembrane receptor CCR5 to enter and infect host cells (Lederman *et al.*, 2006). About 10% of humans of north European ancestry carry a variant called *CCR5*-delta32, in which the gene is disrupted by a deletion (Liu *et al.*, 1996; Lederman *et al.*, 2006). Individuals with one or two copies of the mutant allele are much less susceptible to HIV-1 infection than wild type individuals (Liu *et al.*, 1996; Lederman *et al.*, 2006). In humans, host genes with allelic variants protective against HIV-1 fall into several categories, and may represent HIV co-receptors, immune modifiers (HLA and cytokines) or post-entry retroviral restriction factors (An & Winkler, 2010; Zhao *et al.*, 2012).

No protective variants against KoRV have yet been identified in the koala. Nonetheless, one may consider whether genes with analogous function in the koala currently have (or may develop through mutation) allelic variants that would be protective against KoRV. One may also consider whether some endogenous copies of KoRV may eventually develop a protective role against exogenous KoRV. In either case, aspects of koala biology may be relevant to the development of resistance against KoRV, whether potentially mediated by a protective endogenous KoRV, or by host genetic variants resistant against the virus. Koalas appear to have a low degree of genetic variation, and this low variation appears to have been present in the species for more than a century (Wilmer *et al.*, 1993; Tsangaras *et al.*, 2012). The lack of host genetic variants may limit the diversity of potential retroviral restriction genes, and thus limit the ability of resistance against KoRV to increase over time in the population (May & Anderson, 1979).

Another factor that may affect host-retroviral interactions is limited dispersal or fragmented range of the host (May & Anderson, 1990). The high geographic segregation of mtDNA haplotypes suggests that female koalas may have experienced limited dispersal or that gene flow may have been limited by the fragmentation of species range (Wilmer *et al.*, 1993; Taylor *et al.*, 1997; Houlden *et al.*, 1999; Fowler *et al.*, 2000; Tsangaras *et al.*, 2012; Ávila-Arcos *et al.*, 2013). Isolation of koala populations may have been beneficial in potentially slowing the spread of KoRV from north to south. However, such isolation could also have a strongly negative consequence: in order for a selective sweep to occur, there must be geographic dispersal of the genetic variants that confer fitness (Petit & Excoffier, 2009). Limited dispersal or isolation of populations would limit the degree to which selective sweeps of fitness-promoting variants could occur. Protective effects, whether mediated by endogenous KoRVs that developed a protective role, or mediated by beneficial host genetic variants, could not undergo beneficial selective sweeps in a host population that has limited gene flow (Petit

& Excoffier, 2009). One may even speculate that locally protective variants could potentially be evolving in the koala population separately, but with an inability to improve fitness across the species due to limited geographic dispersal or connectivity (Tack *et al.*, 2012).

KoRV and biomedical research: towards an understanding of koala and human ERVs

Human endogenous retroviruses (HERVs) and related elements comprise ca. 8% of the human genome, a larger proportion than is accounted for by protein-coding genes (Jern & Coffin, 2008). Most HERVs are considered to be non-functional "junk" DNA (Jern & Coffin, 2008). However, recently several HERVs have been established to play a role in human health and disease. For example, the gene *syncytin* plays a functional role in human placental formation (Mi *et al.*, 2000). *Syncytin* is derived from a HERV that entered the germ line of a primate ancestor of the human lineage, since the gene is also present in apes and old world monkeys. The syncytin protein plays a role in formation of the syncytiotrophoblast, a multi-nucleated structure that is vital for normal placentation. Thus, an ERV has been co-opted by its host lineage to play a critical function in the host organism. Interestingly, analogous use of endogenous retroviruses has now been found in rodents, sheep, and other species (Cornelis *et al.*, 2013). Yet the ERVs that play a role in placentation do not derive from a common ancestral invasion of the germ line by the same ERV. Rather, it appears that different ERVs that invaded the germ lines of different mammalian ancestors have been co-opted for placentation across different lineages (Cornelis *et al.*, 2013).

Detrimental long-term effects have also been established for ERVs in various species. Although a role for HERVs has been proposed for many diseases (Voisset *et al.*, 2008), only recently has a direct role in a human disease been established. In Hodgkin's lymphoma in humans, one of the critical steps leading to formation of the disease involves de-repression of an ERV promoter (Lamprecht *et al.*, 2010). Activation of this promoter plays a central role in tumor cell survival (Lamprecht *et al.*, 2010). One reason that it may be difficult to establish a role for ERVs in other diseases is that the human complement of ERVs will be quite different from the ERVs present in biomedical model organisms such as the mouse. The mouse lineage is separated from the human lineage by 85 million years of evolution, involving completely independent invasions of the germ line by ERVs during that time (Johnson & Coffin, 1999; Murphy *et al.*, 2001). Thus diseases caused by ERVs in commonly studied biomedical model organisms may be quite different from those caused by HERVs in humans, and vice versa.

Given that organisms commonly relied upon for biomedical studies may not be directly suitable models for human ERVs, and given that most ERVs, including HERVs, invaded their host germ lines thousands or millions of generations ago, the ongoing invasion of the koala germ line by KoRV may be of great biomedical importance (Hanger *et al.*, 2000; Tarlinton *et al.*, 2005; Fiebig *et al.*, 2006; Oliveira *et al.*, 2006; Stoye, 2006; Tarlinton *et al.*, 2006; Denner, 2007; Tarlinton *et al.*, 2008; Langhammer *et al.*, 2011; Miyazawa *et al.*, 2011; Cui *et al.*, 2012; Denner, 2012; Simmons *et al.*, 2012; Ávila-Arcos *et al.*, 2013; Shojima *et al.*, 2013). The transitioning of KoRV from exogenous retrovirus to endogenous provirus is currently underway, and this represents an excellent, and so far the only, opportunity for studying the process of retroviral germ line invasion prospectively rather than retrospectively (Stoye, 2006; Tarlinton *et al.*, 2006; Tarlinton *et al.*, 2008). This potential utility of koalas and KoRV for understanding the origins of 8% of the human genome should also be seen as potentially beneficial to the koala (Fiebig *et al.*, 2006). Even if some biomedical studies of KoRV have as their primary goal insights into the processes that gave rise to ERVs in humans, any information gained from biomedical studies that increase our understanding of KoRV will necessarily increase our ability to help koalas afflicted with the virus.

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Koala Retrovirus (KoRV): Molecular Biology and Evolution

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Abstract. The koala retrovirus (KoRV) is in transition between occurring as an exogenous retrovirus spread by infection and becoming an endogenous retrovirus spread primarily as part of the host germ line. While up to 10% of mammalian genomes are composed of such endogenous retroviruses (ERVs), KoRV is the only known example of a retrovirus in the process of making this transition. Thus, it presents a singular opportunity to study the host-pathogen interactions involved during retroviral invasion of a vertebrate germ line.

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Overview of KoRV molecular biology

KoRV is most similar genetically to the gibbon ape leukemia virus (GALV). However, biologically, they are quite different. GALV is a highly aggressive oncogenic virus whereas KoRV, while associated with leukemia in koalas, is not as infectious (Hanger *et al*., 2000). Molecular studies of the genetic differences between KoRV and GALV have demonstrated that specific mutations likely account for the decreased pathogenicity of KoRV relative to GALV (Oliveira *et al*., 2006, Oliveira et al., 2007). Thus, replacing important GALV domains with their KoRV homologues decreases the infectivity of the GALV recombinants. Recently, however, an infectious KoRV clone has been developed that, despite its molecular differences with GALV, remains quite capable of infecting and replicating in tissue cultures from many mammalian species (Shojima *et al*., 2013). This KoRV clone provides a novel resource for comparative studies. Since this KoRV clone can infect a wide variety of mammalian cell types, it is not clear why KoRV has only been detected in koalas and is not more widely distributed.

KoRV titres are positively correlated with infection by the bacterial pathogen *Chlamydia*, which has a severe impact on koala health (Tarlinton *et al*., 2008). The interaction between KoRV and *Chlamydia* in terms of koala health needs to be clarified, in order to design appropriate interventions. Currently, research on KoRV and *Chlamydia* occur largely independently of one another, although both would benefit from coordination of efforts.

Genomically, KoRV integration sites vary across infected individuals, most likely KoRV inserts largely at random across the genome of koalas (Hanger *et al*., 2000; Tarlinton *et al*., 2006). In northern Australian koala populations, some copies of KoRV are found at the same locus across individuals, suggesting that the virus has been vertically transmitted as an endogenous retrovirus, i.e., has become part of the germ line. The KoRV genome is highly but variably expressed in tissues of infected individuals, as is common for exogenous and endogenous retroviruses alike (Seifarth *et al*., 2005). Among various other ERVs, expression of endogenous proviruses may evolve to benefit the host species, e.g., through development of novel gene

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function or protection from infection by other retroviruses (Blikstad *et al*., 2008). However, in the case of KoRV, there is currently no evidence of a positive benefit to koalas from the ongoing endogenization of proviruses. Conversely, a detrimental role for KoRV has not been conclusively established either, and the mechanisms by which KoRV may be involved in enabling *Chlamydia* infections or in tumor formation are not well characterized.

Evolution of KoRV

Evolutionary analysis of KoRV suggests that it is most closely related to GALV, more distantly to pig endogenous retroviruses (PERVs), and embedded overall in a clade of retroviruses that include the murine leukemia viruses (MLVs) (Hanger *et al*., 2000). These comprise the gamma-retroviruses, a genus of the Retroviridae, with many members known to cause diseases, particularly cancers. The phylogeny is somewhat surprising as GALV has only been detected in captive or small introduced free-ranging populations of Southeast Asian gibbons, while KoRV is found in both captive and free-ranging Australian koalas (Reitz *et al*., 1979). The host species do not overlap in geographic distribution, which are separated by deep seas and were not connected by land bridges in the past. This would likely rule out the direct transfer of a virus from gibbon to koala, and may suggest transfer from a third species, rodent or perhaps bat. Bats are known to carry retroviruses similar to GALV and KoRV (Cui *et al*., 2012). Rodents, and particularly the diverse species of *Mus* from Southeast Asia, are also potential reservoirs: GALV-like sequences have been detected in *Mus caroli* and *Mus cervicolor* (Lieber *et al*., 1975; Benveniste *et al*., 1977). Modern sequencing efforts have not followed up on this rodent research, conducted nearly 40 years ago. Further progress in understanding the evolutionary origins, diversity and epidemiology of the KoRV/GALV family is of particular interest given the broad diversity of species infected by related viruses and considering that GALV is a pathogenic retrovirus that infects higher primates.

How and when did KoRV appear in the koala population? Until recently it was thought that the ancestor of KoRV may have entered the koala population as recently as ca. 200 years ago, and then spread from northern Australia southward (Tarlinton *et al*., 2008). The geographic spread of KoRV is incomplete since KoRV is ubiquitous among northern koalas but absent in some southern populations. In many northern locations 100% of koalas are positive for KoRV. Until recently it was thought that some parts of the south, and in particular islands off the southern Australian coast, were completely KoRV free. However, recent research suggests that, while low in prevalence, KoRV may be present in most if not all southern populations (Simmons *et al*., 2012). Whether this represents historical or recent introduction of KoRV to southern populations is unclear. In northern koalas, individuals may share the same KoRV integration sites (Hanger *et al*., 2000; Tarlinton *et al*., 2006). This suggests that KoRV, while persisting as an exogenous retrovirus, also exists as an endogenous retrovirus. Of course, since some koalas are completely KoRV-free, there are not yet any fixed ERVs, those found at the same chromosomal location in all members of a species. This lack of fixation is an indication that the process of retroviral endogenization by KoRV in koalas is a recent one (relative to many ERVs found in other taxa) that is still underway.

Recent analysis by our group suggests that while the overall evolutionary trajectory postulated for KoRV is largely correct, the time frame appears to have been underestimated (Ávila-Arcos *et al*., 2013). Using museum samples dating from the late 1800s to present, we demonstrated using next generation sequencing (NGS) that KoRV was widespread in northern Australian koalas by the 1800s. Since KoRV was already ubiquitous among koalas in northern Australia by the late 1800s (close to the previously postulated time at which the virus first infected koalas), it seems likely that the initial infection of koalas by KoRV occurred far earlier than 200 years ago.

We also found that evolution of the provirus across this time has been extremely slow (Ávila-Arcos *et al*., 2013). Only minor mutations that appeared to be individual-specific were found in the region of *env* coding for the receptor-binding domain. The receptor binding domain of the virus is exposed to the host immune system and is thus under the most pressure to make compensatory changes. The slow evolutionary rate of KoRV suggests that it has been under limited pressure to evolve, and also suggests that koalas have been affected for an extended period of time by harmful KoRV strains that reduce host fitness. We recently demonstrated by examining mitochondrial DNA variation from museum samples that genetic diversity in koalas has been low for more than a century, and it is possible that KoRV pathogenicity has persisted for this time due to the limited genetic diversity of the host species, which could preclude substantial increases in host fitness in response to KoRV (Tsangaras *et al*., 2012). Selective sweeps of resistance to KoRV would not be possible if koalas lack the genetic variation necessary to mediate differences in fitness. On a more positive note, the slow evolutionary rate of KoRV may also suggest that vaccines targeting viral proteins face limited diversity in the targeted proteins, increasing the chance that vaccinated koalas may be successfully protected from infection.

Looking forward

For retroviruses such as KoRV that can form quasi-species and that exist in the genome at high copy number, complex analytical tools may be required. Analytical problems are compounded for historical samples since DNA damage can introduce substantial amounts of artifactual variation. As described in the previous section, NGS can provide novel data and insights; however, new methods are showing even greater promise.

Hybridization capture is a method whereby PCR products or synthesized oligonucleotides (similar to PCR primers) can be used as "baits" to "fish" out or capture related sequences from genomic DNA or reverse transcribed RNA libraries (Maricic *et al*., 2010). Such methods have several advantages over PCR amplification, especially as applied to retroviruses or to ancient DNA (Fig. 1). First, while PCR is very sensitive, it is susceptible to false negatives due to primer-target mismatch. Second, for PCR to work, DNA molecules must be present that are the size of the target amplicon, typically at least 50 bp and often much larger. By contrast, the PCR products or oligonucleotides used for enrichment can bind their targets even if the target DNA is much shorter or contains mismatches. This is important in particular for retroviruses since different virus copies may be quite divergent. Also, short target DNA fragments typical of historical samples (often no larger than 100 bp and sometimes under 20 bp) can be readily retrieved by hybridization. The number of targets that can be "baited" in a given experiment is effectively unlimited. Coupled with NGS, many kilobases or tens of kilobases of target DNA

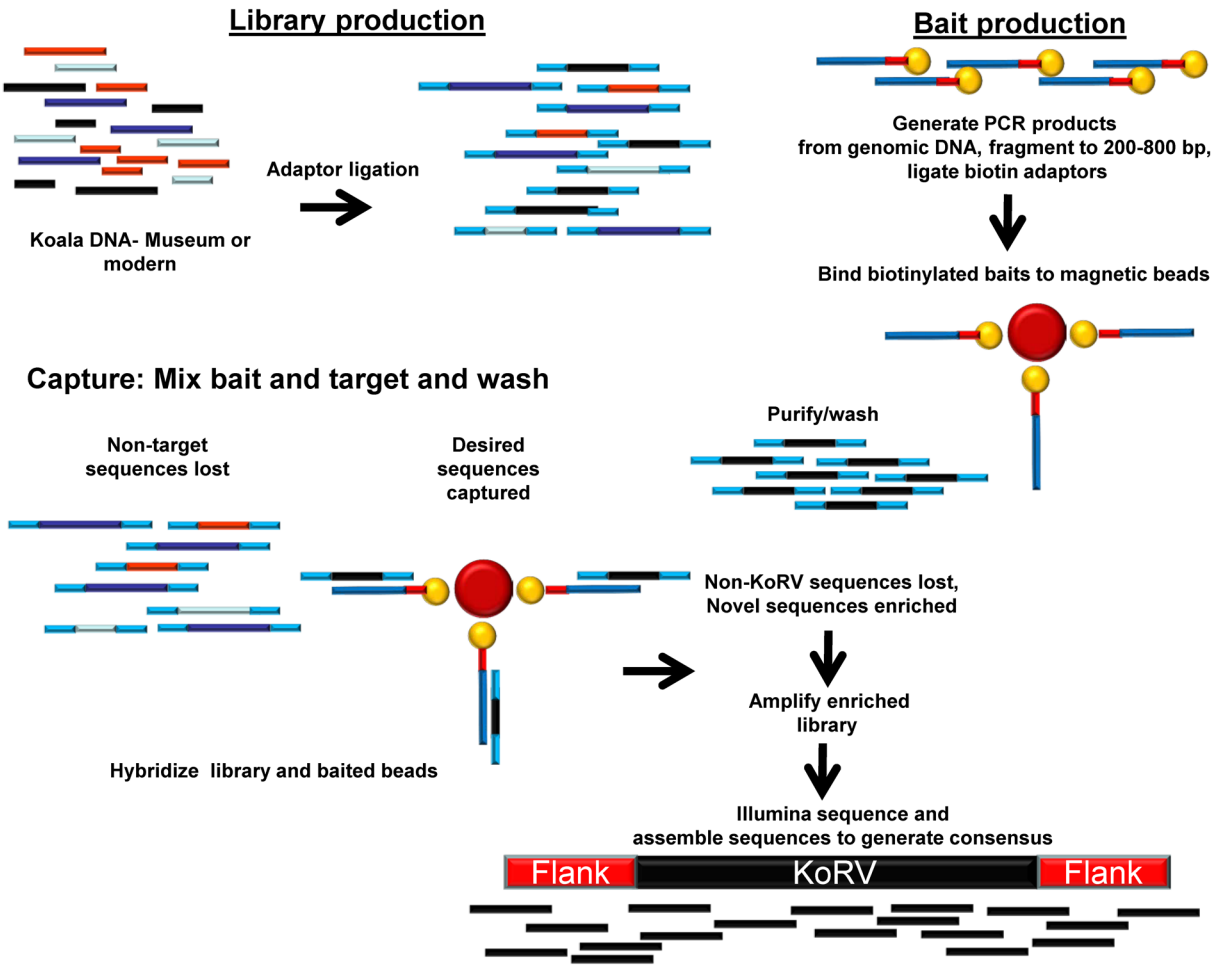

Figure 1. An illustration of the steps involved in hybridization capture. Library preparation is standardized and requires DNA fragments of limited size. For modern samples, DNA must be fragmented to fit the minimum and maximum requirements of the NGS library insert size recommended. For ancient or historical DNA, fragmentation is often not necessary because the DNA is often highly degraded. Adaptors (shown as light blue) are ligated on both sides of each DNA strand. These will be used to amplify the libraries in later steps and, when multiple samples are used, to also identify each library, so that sequences can be separated by sample using the identifiers in a post-sequencing bioinformatics routine. "Bait" preparation involves generating PCR products for the desired targets. It can involve amplifying a long fragment and then shearing to ca. 200 bp, or generating multiple short amplicons. Regardless of how the bait is generated, the bait sequences will be ligated to a biotinylated adaptor (yellow circles) attached to an adaptor sequence (red). The biotin linked baits are then attached to streptavidin-coated magnetic beads. Baits and target libraries are then hybridized together. DNA in the library with sequences complementary to the baits will bind to them; non-matching sequences will not. The target-bait hybridized beads are then isolated using a magnet while the unhybridized DNA is washed off. Using heat or NaOH, the captured strands are separated from the baits resulting in a highly purified library representing the desired target, which is then sequenced using next-generation methods. Sequence reads are aligned to a reference sequence; in this case, KoRV.

can be retrieved during a single experiment. By appending a short fragment of DNA containing a specimen identifier to each set of captured DNA, multiple samples can be processed in one NGS run. Illumina and 454 GS FLX technologies provide ample read length and high throughput, suitable for a broad variety of experimental needs. Such techniques have been extremely successful for ancient DNA and virological studies, including the sequencing of *Yersina pestis* genomes from 500 year old plague pits (Schuenemann *et al*., 2011), and identifying variation and integration sites for the Merkel cell polyomavirus in formalin fixed tissues (Duncavage *et al*., 2011).

We are applying these methods to investigate the evolution of KoRV. These are proving superior to our previous analysis by PCR and NGS and have yielded full KoRV genomes from modern and museum specimens and have determined integration site variation (Tsangaras *et al*., 2014). These methods can reveal the full palette of variation at any given site of the KoRV genome in any individual. KoRV present across individuals at the same locus, which likely represent endogenous proviruses, can be examined for their presence or absence in museum samples and further investigated. We have also applied the technique to rodents that could harbor relatives of KoRV and GALV with promising leads regarding the reservoirs for some known GALV strains. As these methods are in their infancy, they will develop further and will enable studies of KoRV molecular evolution to advance rapidly.

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Koala Retrovirus in Free-Ranging Populations—Prevalence

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Abstract. The prevalence of koala retrovirus (KoRV) provirus (DNA) and the average number of proviral insertions per cell vary in different free-ranging koala *(Phascolarctos cinereus)* populations across Australia. Populations in the northern states of Queensland and New South Wales have 100% proviral prevalence and mean proviral copy number of 140–165 per cell. In contrast, the proviral prevalence in the southern states of Victoria and South Australia differs among populations, with a mean prevalence in these states' mainland populations of 73% and 38%, respectively and with the prevalence on southern island populations ranging from 0–50%. The proviral load in southern populations is comparatively low, with some populations having an average of less than 1 proviral copy per cell. The KoRV RNA load in plasma shows a similar discordance between northern and southern populations, with consistently high loads in northern koalas (103 to 1010 RNA copies per ml plasma), and loads ranging from 0 to 102 copies per ml in southern KoRV provirus-positive koalas. The variation in KoRV proviral prevalence and the disparity in proviral and viral loads between northern and southern koalas may reflect different types of infection in the two populations (endogenous versus exogenous). Alternatively, it is possible that KoRV has been present for a longer time period in northern populations resulting in differences in the host-virus relationship.

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Koala retrovirus (KoRV) is a gammaretrovirus of koalas *(Phascolarctos cinereus)* that possesses features of both endogenous and exogenous viruses. In previous work, we demonstrated that KoRV is truly an endogenous virus in koalas in south-east Queensland, with proviral DNA present in every animal tested and also present in single sperm cells. We also showed evidence of specific proviral insertion inheritance in Queensland koalas (Tarlinton *et al.*, 2006). However, KoRV is clearly not endogenous in all koala populations in Australia because our early work demonstrated mixed KoRV presence in some southern populations (Tarlinton *et al.*, 2006). Despite its endogenous nature in Queensland koalas, KoRV also displays exogenous virus characteristics in these populations, with high levels of viral RNA present in the blood of every animal tested, indicating active transcription of the KoRV proviral elements (Tarlinton *et al.*, 2005). There is also considerable variation in the number and sites of KoRV proviral insertions in individual koalas, which again is not typical for an endogenous virus where the conservation of a proviral

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integration pattern is expected (Tarlinton *et al.*, 2006). Taken together, these findings suggest that the virus is behaving as both an exogenous and endogenous virus in different koala populations, with transmission of the virus possibly occurring by inherited and/or horizontal routes.

KoRV is closely related to gibbon ape leukaemia virus (GALV), a pathogenic exogenous virus of gibbons (Hanger *et al.*, 2000; Tarlinton *et al.*, 2008). The genetic similarity between KoRV and GALV led to speculation that the two viruses diverged only recently (Bromham, 2002). The sequence similarity between KoRV and GALV is so close across the complete proviral genome that either recent cross-species transmission of virus between koalas and gibbons, or transmission from an intermediate host species is likely (Hanger *et al.*, 2000; Martin *et al.*, 1999). Given that koalas and gibbons do not exist in the same geographical locations in nature and that to date GALV has only been detected in captive gibbons, direct cross-species transmission in the wild appears unlikely. The most probable explanation for the close genetic similarity between KoRV and GALV is that they each were derived from a common virus hosted by a third species of animal whose distribution encompasses that of both gibbons and koalas.

To better understand the endogenous/exogenous nature of KoRV infection in different koala populations, and potentially to gain insight into the origins of the virus, we are conducting on-going studies into the prevalence of KoRV infection across the species' range, including investigation of KoRV proviral DNA load and viral RNA load in these different populations. The samples tested in our studies are collected from a range of sources including koalas presented to veterinary clinics because of illness or trauma, koalas trapped in other research projects, archival samples stored by other researchers and koalas undergoing sterilisation procedures on Kangaroo Island. Related to the diversity in source of samples, the sample type also covered a spectrum including blood, internal organs from euthanized animals, and ear punch biopsies.

Methods used to detect and quantify KoRV proviral DNA and viral RNA comprise standard PCR using *pol* gene primers (Tarlinton *et al.*, 2006), nested PCR using internal primers (Simmons *et al.*, 2012), real-time PCR (qPCR) (Tarlinton *et al.*, 2005), and reverse transcriptase real-time PCR (RT-qPCR).

Prevalence of KoRV provirus in free-ranging koala populations

The prevalence of KoRV provirus in different koala populations ranges from 100% in the northern states of Queensland and New South Wales (NSW) to 0% on one of the southern off-shore islands (Phillip Island) (Simmons *et al.*, 2012). Previously published and recent work has shown that the prevalence in populations on mainland Victoria and South Australia falls between these two extremes, with a mean prevalence on mainland Victoria and South Australia of 73% and 38%, respectively (Simmons *et al.*, 2012; Jones, unpublished data). The prevalence on other southern islands varied considerably, with 50% of koalas tested being provirus-positive on Snake Island, 35% on Raymond Island, 21% on French Island and 15% on Kangaroo Island (in 2007), although the small number of samples tested from some of these islands limits the strength of these data (Simmons *et al.*, 2012). In contrast, the KoRV proviral prevalence on islands off the coast of Queensland is 100% (Jones, unpublished data).

To investigate an apparent change in the dynamics of KoRV infection on Kangaroo Island, we are conducting a temporal and spatial study of KoRV proviral prevalence on the island, which is off the coast of the state of South Australia. In our initial study of blood samples collected from koalas on the island in 2004, none of 26 koalas was provirus-positive (Tarlinton *et al.*, 2006). Twenty-four of 162 (15%) blood samples collected in 2007 were provirus-positive (Simmons *et al*., 2012), and 19 of 50 (38%) and 10 of 38 (26%) samples collected in 2009 and 2011, respectively were provirus-positive (Jones, unpublished data). There does not appear to be a clear geographic segregation of KoRV provirus-positive and –negative animals on Kangaroo Island. However, koalas in the central region of the island are over-represented in each of the four years of our sample collection, thus interpretation of these data must be guarded. The samples used in our study have been collected from koalas caught for a government-funded sterilisation program, which aims to address the over-abundance of the introduced koala population on the island. In the years we obtained samples, this program was focused on the central region of the island, with only limited trapping activities in the western and eastern parts of the island. To date, the small number of animals tested from the western part of the island has been KoRV provirus-negative, but further samples from that part of the island should be tested to confirm these findings.

KoRV proviral and viral loads

We established methods to quantify KoRV proviral DNA and viral RNA loads in cells and plasma, respectively. Initially we used these methods to investigate relationships between either proviral or viral load and disease in koalas, in particular lymphoid neoplasia and chlamydial disease. We found a significant association between KoRV viral load in plasma and the presence of lymphoid neoplasia (Tarlinton *et al.*, 2005). Although there was a trend towards increasing viral load and severity of chlamydiosis, the relationship was not significant.

Using qPCR, we then investigated the levels of KoRV proviral load amongst different koala populations in Australia. The proviral copy number per cell was estimated either from the quantification of DNA concentration in the sample or from comparison to beta-actin copy number (Delta-Delta Ct). Using the first of these approaches, the proviral copy number per cell from DNA extracted from ear punch biopsies of Queensland koalas was markedly higher than that of koalas in southern states, with a mean of 165 copies per cell in Queensland koalas compared to means of 1.5, 0.00153 and 0.000129 copies per cell in three different populations of Victorian koalas (Simmons *et al.*, 2012). Similarly, using the second approach, the means of proviral copies per cell from DNA extracted from blood cell pellets varied from 140 in Queensland koalas, 10 in Kangaroo Island koalas and 1.3 in South Australian mainland koalas (Jones, unpublished data).

KoRV viral RNA levels were determined using RT-qPCR on plasma samples. The viral load in plasma of Queensland koalas is consistently high, ranging from 4.3×10^3 to 8.2×10^{10} copies/ml plasma (Simmons, 2011). In contrast, plasma samples from koalas on Kangaroo Island range from only 1.1×10^2 to 4.3×10^2 copies/ml plasma (Jones, unpublished data) and not all KoRV provirus-positive koalas have detectable viral RNA in plasma.

Discussion

Our data clearly show a marked difference in KoRV proviral prevalence between the northern states of Queensland and NSW and the southern states of Victoria and South Australia. The proviral prevalence declines even further on the southern off-shore islands. Possible interpretations of these data include the northern introduction of the virus with subsequent spread to the south, an inherent genetic resistance to infection or to endogenization of the virus in southern koala populations, or the absence of some kind of environmental factor or vector in the south that limits transmission of the virus. With the current state of knowledge on KoRV, there is insufficient evidence to provide convincing support for any one of these possible interpretations over others.

The large variation between KoRV proviral load of northern koalas in comparison to southern koalas may imply a different type of infection between the two populations. The consistently high copy number of KoRV provirus in individual northern koalas is indicative of endogenous infection, and confirms our earlier evidence for likely endogenous infection of animals in this region. In contrast, the low proviral load in southern populations, with some koalas having less than one provirus copy per cell is clearly not consistent with endogenous infection and more likely reflects exogenous transmission of the virus in these regions.

Despite the probable endogenous infection of Queensland koalas, the KoRV viral RNA load in plasma of these koalas is very high, suggesting that these animals have little control over KoRV transcription and viral replication. In contrast, our findings from the small number of southern koalas on Kangaroo Island tested to date reveal relatively low levels of KoRV RNA in plasma, with some provirus-positive individuals having no detectable viral RNA in their plasma. Coupled with these findings of comparatively low proviral and viral loads, we have also detected a genetic variant of KoRV in koalas on Kangaroo Island, which has a different variable region A (VRA) of the receptor binding domain of the viral *env* gene. Since the VRA is thought to provide specificity for cell surface receptor binding and viral entry, the *env* variant may vary in receptor binding affinity or indeed receptor usage compared to the KoRV A variant found in Queensland. It is unknown whether this genetic variation of the virus is involved in producing the different manifestations of KoRV infection and prevalences between northern and southern populations. Recent studies have reported on similar *env* variants in koalas from zoos in the US (Xu *et al.*, 2013) and Japan (Shojima *et al.*, 2013; Shimode *et al.*, 2014), with the *env* variant from Kangaroo Island showing closest identity to the variant designated KoRV-C from a koala in the Kobe Zoo (Shimode *et al.*, 2014; Young, 2014). Further research is required to better understand the distribution, prevalence and pathogenicity of these *env* variants of KoRV.

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Disease in Wild Koalas *(Phascolarctos cinereus)* with Possible Koala Retrovirus Involvement

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Abstract. A wide range of serious, and oftentimes fatal, conditions has been observed in both free-living and captive populations of koalas *(Phascolarctos cinereus)* and are attributed, perhaps prematurely, to the koala retrovirus (KoRV). These maladies include lymphoma, leukaemia, and other bone marrow conditions, and the so-called koala AIDS. A variety of other conditions that involve disordered growth of cells and tissues, altered or inappropriate immune responses, and degenerative conditions may also be consequences of insertional mutagenesis, or other pathogenic mechanisms associated with KoRV infection. The list of potential KoRV-associated pathologies continues to grow, as more thorough and consistent approaches to clinical assessment and diagnosis are applied to wild and captive koalas.

Hanger, Jon J., and Jo Loader. 2014. Disease in wild koalas *(Phascolarctos cinereus)* with possible koala retrovirus involvement. In *The Koala and its Retroviruses: Implications for Sustainability and Survival*, ed. Geoffrey W. Pye, Rebecca N. Johnson and Alex D. Greenwood. *Technical Reports of the Australian Museum, Online* 24: 19–29.

This paper aims to briefly describe a selection of well-recognized and newly-observed conditions that may have KoRV as a contributing factor. For most, however, the link with KoRV is evidentially non-existent, and its role in those diseases purely speculative. However, they are listed here to give KoRV researchers, particularly those based in laboratories, a fuller picture of the clinical spectrum of disorders afflicting koalas, and perhaps some guidance on future research directions. Some of these conditions, in the fullness of time, may have their definitive aetiologies and pathogenesis better illuminated as a result.

The evidence for the involvement of a retrovirus in leukaemia in koalas began building with the discovery and reporting of virus particles in the bone marrow of a leukaemic koala in 1988 (Canfield *et al.,* 1988), and the recognition of a spectrum of conditions in koalas similar to that observed in FeLV-infected cats (Hanger, 2009). A full-length KoRV genome sequence was reported in 2000 (Hanger *et al.*, 2000). While it is tempting to conclude that KoRV is responsible for leukaemia, lymphoma, and related diseases in koalas, causation has not yet been proven. Certainly KoRV has characteristics found in pathogenic gammaretroviruses, including the immunosuppressive domain of the transmembrane portion of the envelope protein (Fiebig *et al.,* 2006). The evidence for KoRV pathogenicity in koalas is building, but not unequivocal.

This paper describes a wide range of diseases and syndromes observed in wild koalas, mainly in Queensland, which may be caused by disruption of normal cellular function and regulation by KoRV. It is important to remember that none has been conclusively causally linked to KoRV infection. These diseases have been included because they represent disordered growth of tissues, either as neoplasia, or benign conditions, or they are associated with putative disruption to normal cellular function, for example, the koala "AIDS" condition. Also included are some immune-mediated conditions, which may be associated with immune dysfunction or dysregulation by KoRV, or perhaps other factors.

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Figure 1. Cranial osteochondroma.

Disorders of growth—Neoplasia

Leukaemia

The leukaemias are a group of conditions that include the haematopoietic malignancies and, by definition, arise in the bone marrow. The classical forms of leukaemia are associated with the presence of malignant cells in the bloodstream, but non-leukaemic (aleukaemic) forms occur, in which there are no, or minimal, malignant cells in the circulation. Leukaemias may arise from abnormal early multipotential progenitor cells or from more differentiated progenitor cells, but the phenotype of the cells will depend upon their ability to undergo further differentiation or, in the case of unipotential stem cell transformation, the lineage. Leukaemias may be of lymphoid or myeloid origin; and within the myeloid group may be further characterized as erythroid, granulocytic, megakaryocytic, or monocytic, depending upon the recognition of lineage (Robbins, 1974). Occasionally, leukaemias are encountered that are biphenotypic, in which leukaemic cells may express myeloid and/or lymphoid markers, but are derived from the same abnormal progenitor cell clone, or more rarely from separate clones (Ganesan, 1995; Lichtman, 1995).

A variety of types of leukaemia have been observed in koalas. Lymphoid leukaemia appears to be the most common, and is, as often as not, associated with solid tumours of lymphoid origin. Myeloid and erythroid lineage leukaemias have also been seen, although definitive identification of cell lineage (using cytochemical or immuno-cytological techniques) has not been attempted for most. A leukaemia of megakaryocyte-like morphology was noted

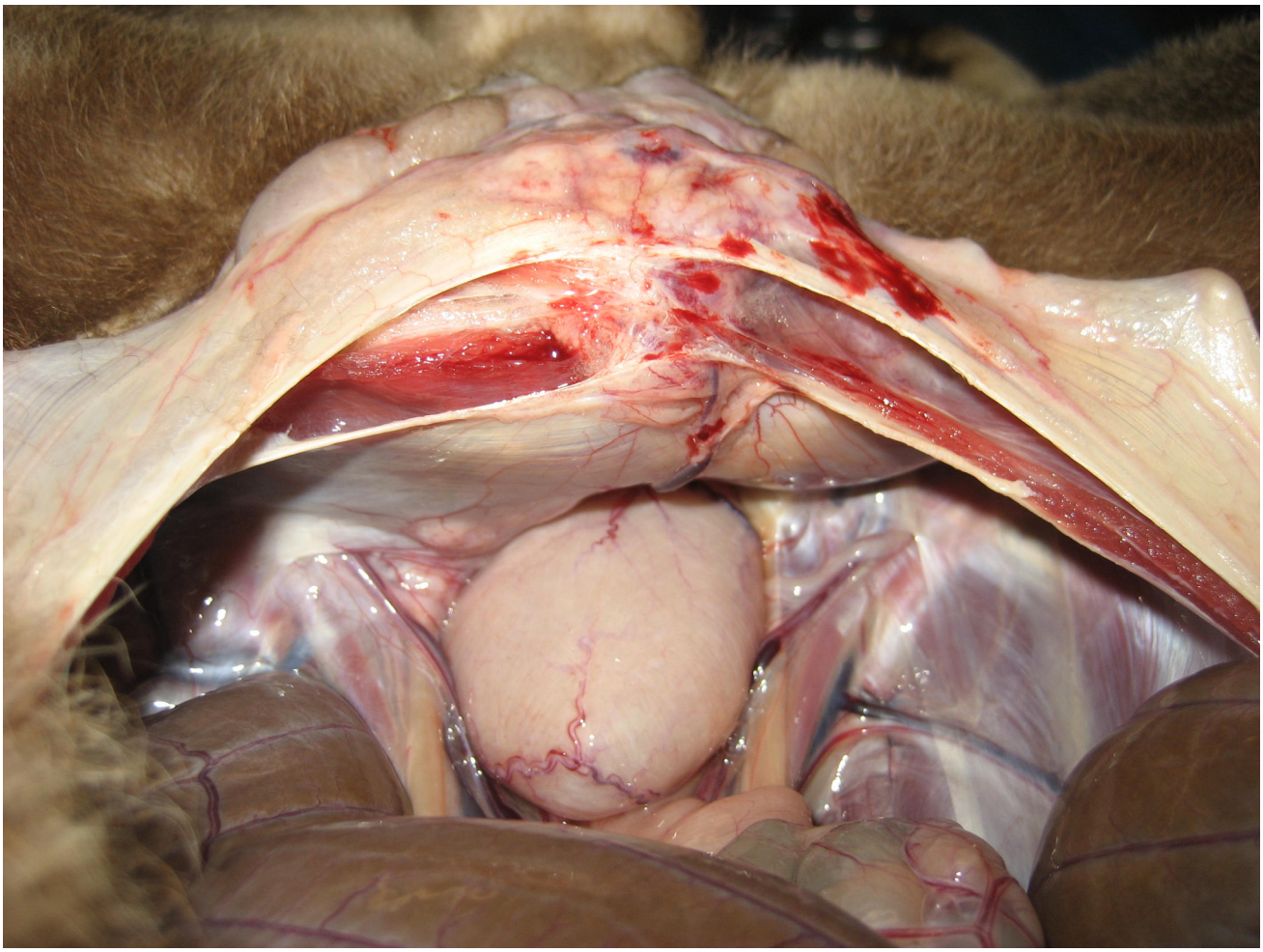

Figure 2. Pelvic osteochondroma.

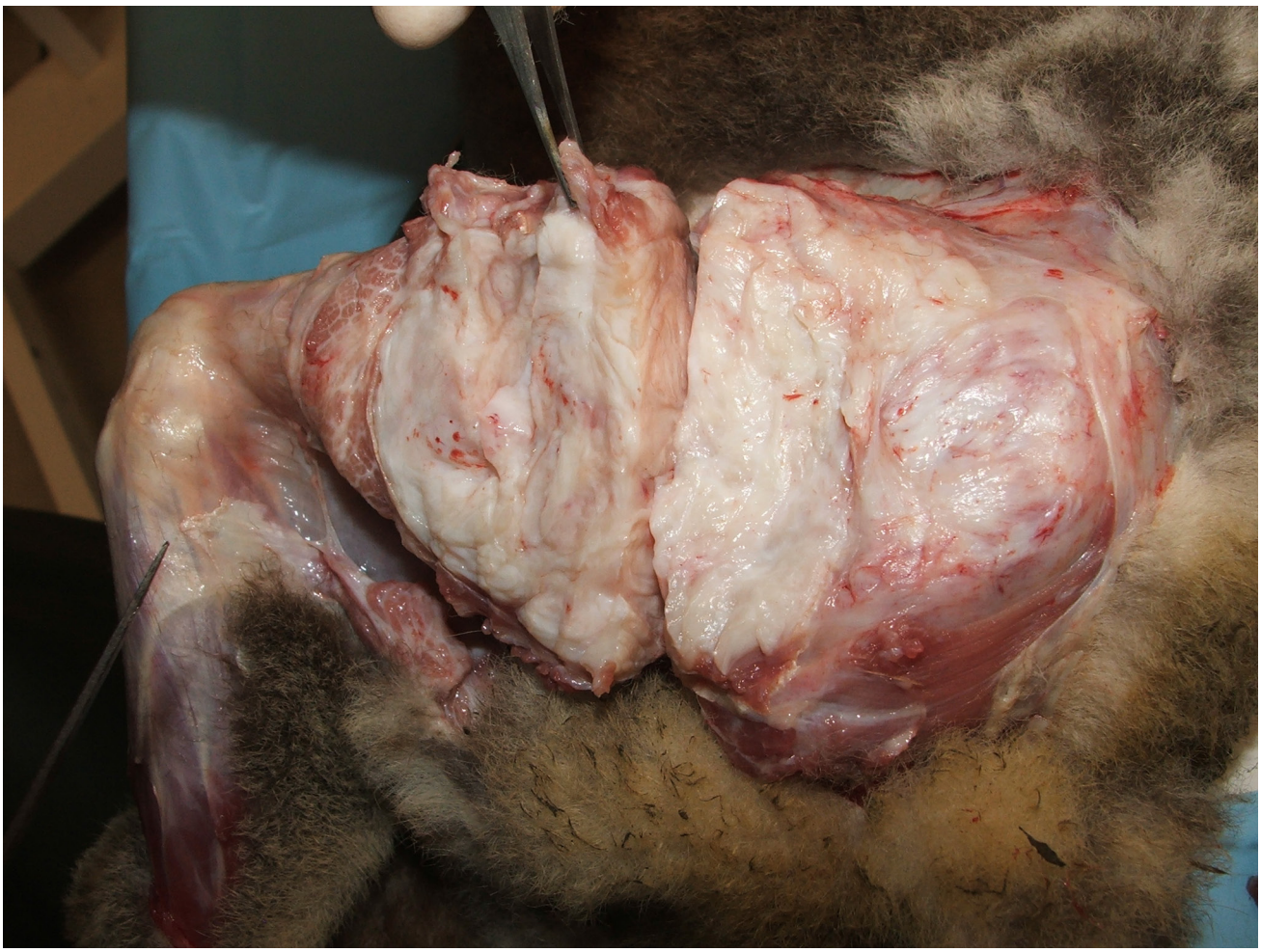

Figure 3. Fibrosarcoma on the thigh of a koala.

in a wild koala from the Gold Coast, which was euthanized because of paralysis associated with spinal infarction. Cells resembling immature megakaryocytes were observed in a wide range of tissues. Some leukaemias are quite obvious on blood smears, others (the "aleukaemic" forms) are not. In these cases, some white cells may appear atypical and the diagnosis must be based on bone marrow cytology. Leukaemias are often associated with infiltration of a wide range of organs and tissues, including brain and spinal cord.

Lymphoma

The term *lymphoma* refers to a malignant proliferation of cells of the lymphoid lineage, and may also be referred to as *lymphosarcoma*. The distinction between lymphoid leukaemia and lymphoma is one of definition: the lymphoid leukaemias arise from clonal expansion of a progenitor cell in the marrow, whereas the lymphomas arise from cells in the other lymphoid tissues. However, in both animals and humans, lymphoma may have a leukaemic phase, and the distinction does not necessarily or consistently define separate clinical entities (Magrath, 1995).

Lymphomas may be singular or multiple solid tumours affecting all lymphoid tissues, or specific subsets, for example: abdominal lymphoid tissues only, peripheral lymph nodes only. Isolated tumours of the thymus have been observed. The tumours are generally firm, pale, off-white tumours, often with areas of haemorrhage and necrosis. Grossly the tumours are often well demarcated, but histologically the margins show invasion into surrounding tissues. Abdominal lymphoma is often not associated with enlargement of peripheral lymph nodes. Ascites is usually present and the fluid contains high numbers of neoplastic lymphoid cells, and is often blood-tinged. Serosal surfaces may be variably affected by a coating of nodular tumour.

Osteochondroma

Osteochondromas are large, usually single, tumours of mixed cartilage and bone that most commonly in koalas affect the bones of the head (Fig. 1) (Blanshard, 1994; Canfield *et al.*, 1987; Sutton, 1986). However, they also occur in the pelvis, ribs, clavicles, and long bones (Hanger *et al.*, 2003). Histologically, these tumours are quite variable, with areas of quite orderly growth of cartilage and bone and other areas with disorderly growth and characteristics of malignancy. Some cases of osteochondroma have crossed the acetabular joint, causing growths on both the pelvic bones and proximal femur. The tumours tend to be expansive, rather than infiltrative, and cause clinical signs referable to displacement, compression, and impingement on organs, tissues, and their functions (Ladds, 2009). The tumours are relatively slow-growing, with one tumour affecting the pelvis of a koala expanding by 10–15% (diameter) every two weeks (Fig. 2).

Fibrosarcoma

One fibrosarcoma was observed in a captive koala at, or close to, a site of vaccination for *Bordetella*. The poorly demarcated and infiltrative tumour affected the skin and subcutis in the interscapular region, and contained cavities containing viscous myxomatous fluid. It was diagnosed histologically as a malignant fibrous tumour, but resolved spontaneously in the koala. The formation of vaccination-

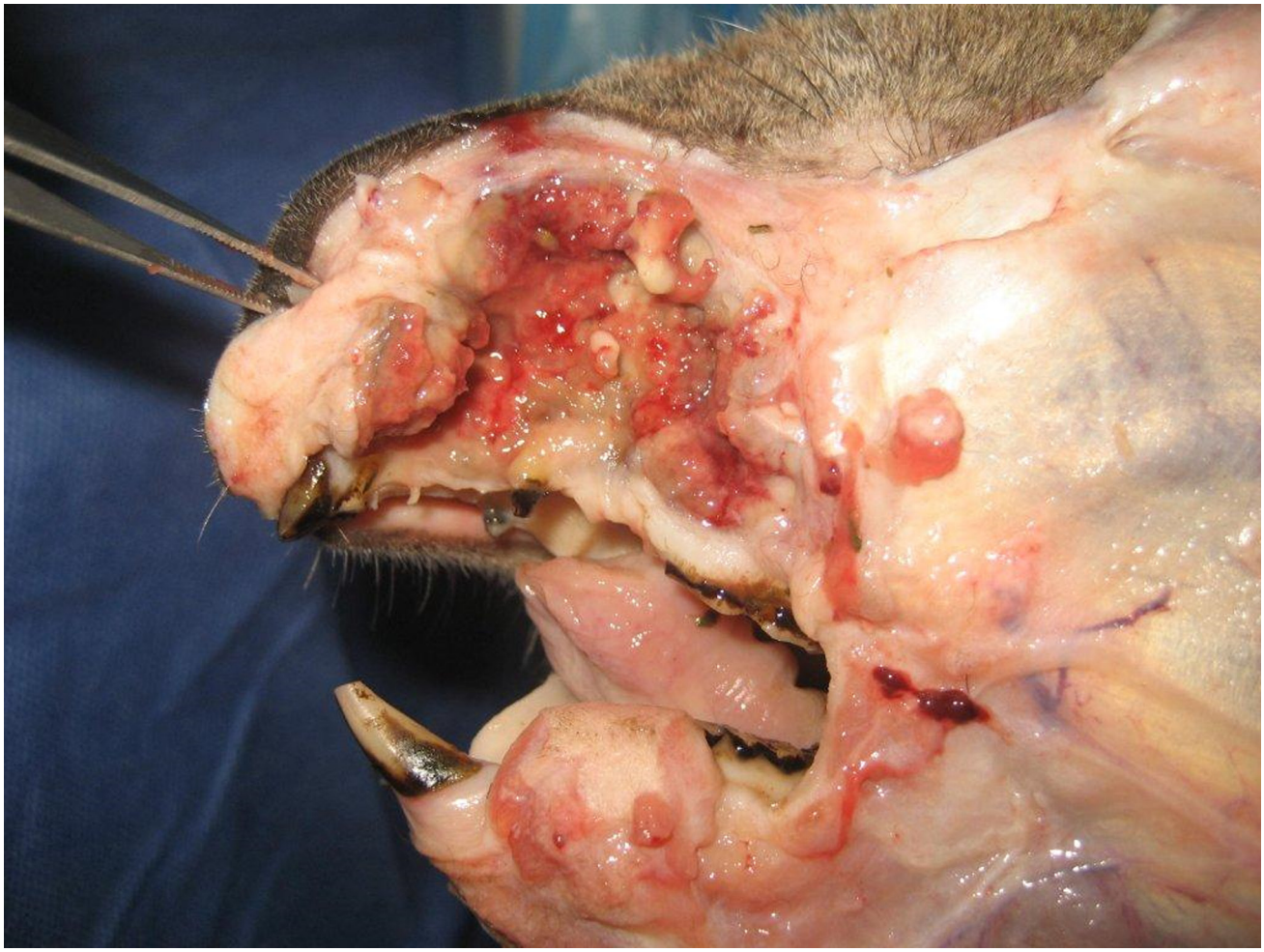

Figure 4. Maxillary fibrosarcoma.

site fibrosarcomas is a well-recognised phenomenon in cats (Kitchell, 2005). In most other cases observed in koalas, the tumour has arisen spontaneously or from an unknown trigger. These tumours tend to be aggressive, rapidly-growing, and infiltrative; affecting a wide range of organs and tissues (Figs. 3 and 4).

Mesothelioma

Mesothelioma in koalas is most commonly a diffuse malignant nodular tumour affecting the abdominal surfaces (e.g., peritoneum, mesentery, and gastro-splenic ligament) or pleura. It results in massive ascites with thick, blood-tinged, viscous fluid, with a consistency similar to joint fluid. Affected koalas present with gross distension of the abdomen, often in poor body condition, and sometimes with dyspnoea. Grossly, both the parietal and visceral peritoneum (serosal surfaces) is covered in small, red nodules with a glistening, slimy surface. Ante-mortem diagnosis is based on the finding of viscous, blood-tinged pleural or abdominal fluid, which strings out significantly when dripped from the tip of a needle or hub of a syringe (Fig. 5). Cytologically, the cells resemble large, atypical, and pleiomorphic mesothelial cells, often with characteristic eosinophilic material apparent in cell clusters (Fig. 6).

Other tumours

A variety of other tumours has been observed in koalas, including squamous cell, renal and sebaceous carcinomas, mammary adeno-carcinomas, and poorly defined neoplastic-like conditions (Blanshard, 1994; Ladds, 2009). One particularly interesting case occurred in a captive koala at the site of a healed fight wound to the right shoulder. The tumour developed in, or near, the periosteum of the proximal humerus and, at the time of euthanasia of the koala, had evolved into a poorly differentiated, pleiomorphic sarcoma containing numerous anaplastic spindle-shaped cells and frequent giant cells. The original lesion radiographically resembled a minor periosteal reaction, presumably in response to injury from the bite wound from another male koala. As the tumour developed, biopsy and cytology submissions to a veterinary pathology laboratory returned diagnoses of “non-suppurative fibrosing cellulitis”, “pyogranulomatous panniculitis”, “granulomatous osteomyelitis”, “anaplastic neoplasm” and, at necropsy, “probable sarcoma” of indeterminate lineage. A 4-cm diameter tumour of similar gross and histological morphology had also developed in the thigh, where the koala had received numerous antibiotic and analgesic injections. This interesting tumour certainly had features of chronic, granulomatous, and fibrosing inflammation and also sarcomatous malignancy, but never received the benefit of a consensus on its definitive histological diagnosis amongst the pathologists examining it. Whether it was a true neoplasm, arising from clonal expansion of a single abnormal cell, or a non-clonal, but unregulated/dysregulated, inflammatory response made little difference to the koala. The tumour acted clinically like a malignant neoplasm and caused sufficient pain and debility to the koala to warrant eventual euthanasia (Cumming, 2008).

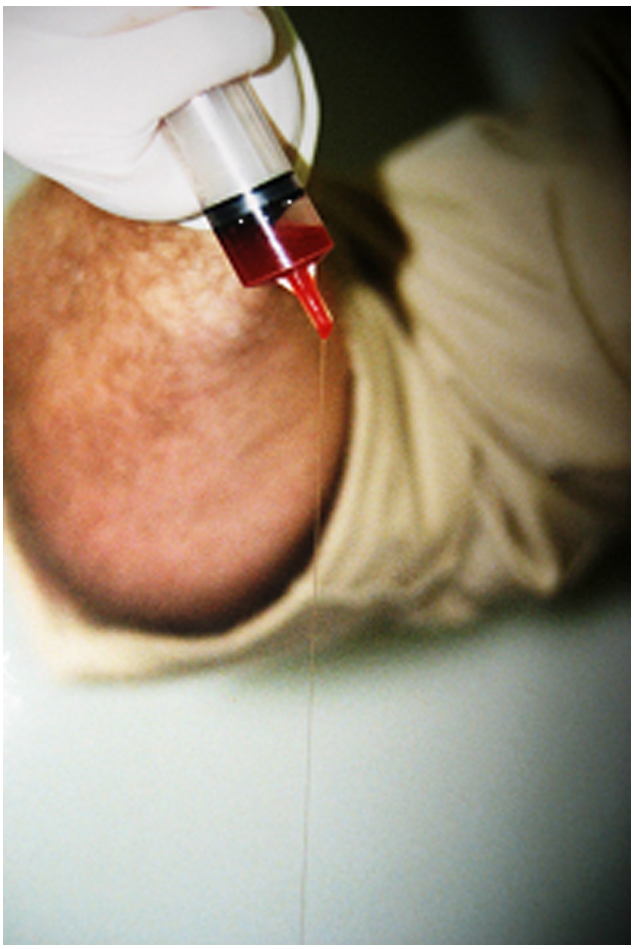

Figure 5. Viscous mesothelioma ascites fluid.

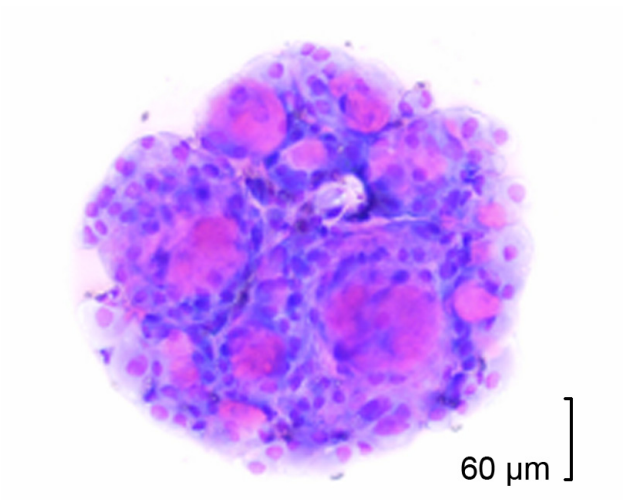

Figure 6. Mesothelioma cytology—showing distinctive eosinophilic material.

Myelodysplasia

Myelodysplastic conditions and myeloid leukaemias are seen occasionally in koalas, but at a lower frequency than lymphoid leukaemia. These cases may be detected by the finding of slightly unusual white cells in blood smears and cytopaenias. However, blood smears may be essentially normal, or have subtle cytopaenias. Anaemia associated with chronic inflammatory disease, and other causes of bone marrow suppression should be ruled out prior to making a diagnosis of primary marrow disease or myelodysplasia. Examination of bone marrow smears, collected from the iliac crest, may reveal hyper, or hypocellularity of the marrow, with atypical cells present or abnormal maturation of haematopoietic cells. In koalas, the distinction between myeloid leukaemia and myelodysplasia is possibly arbitrary, as both conditions are part of a spectrum of disorders that are probably causally and mechanistically linked. Abnormal blood cells may or may not be present in the circulation, and it is likely that myelodysplasia, in cases that survive long enough, may progress to leukaemia.

Benign growth disorders

Sebaceous hyperplasia/adenomatosis, causing generalised and widespread, greasy, multilobular, cutaneous nodules mainly in the ventral skin and pouch (Fig. 7), has been observed in a number of koalas. These small nodules are benign growths of sebaceous gland tissue, the cause of which is unknown.

Plantar and palmar hyperkeratosis, and focal papillary hyperkeratosis

Horn-like, hard, cutaneous growths of the plantar and palmar skin (Fig. 8) have been observed occasionally in koalas and, in some cases, the koalas were concurrently affected by lymphoma or other bone marrow conditions (Canfield *et al.*, 1992; Hanger, 2000). The pathogenesis in koalas may be similar to that occurring in FeLV-affected cats (Muller *et al.*, 1989). A less severe, focal papillary hyperkeratosis has been observed in other koalas: one at the interscapular region, the others on the palmar/plantar skin. It is possible that the newly-discovered koala papillomaviruses are involved in the pathogenesis of these lesions and perhaps KoRV interferes with the normal immune response, allowing more severe lesions to develop. One study found a prevalence of papillomavirus of 10/72 (14%) of koalas swabbed (Antonsson & McMillan, 2006).

Polycystic kidney disease

Unilateral polycystic kidney disease has been observed in one koala, whose other kidney was sonographically and grossly normal. The affected kidney was extensively affected by tubular hyperplasia and cyst formation, which replaced the normal architecture almost to the point of completeness (Fig. 9). Histologically, there were occasional small wedges of normal renal tissue between the large areas of tubular hyperplasia and cysts. There were also occasional foci of mononuclear infiltration and frequent accumulations of what appeared to be neutrophils in and around hyperplastic tubules. The histological changes were entirely consistent with the diagnosis of polycystic kidney disease. The causes of this condition in other species (including humans and cats) commonly have a genetic basis, with 90% of human cases caused by an autosomal dominant genetic defect. Unilateral renal cystic disease in humans is a recognised, but rare disease, in which the affected kidney shows changes histologically indistinguishable from autosomal-dominant polycystic kidney disease (Wilson, 2004).

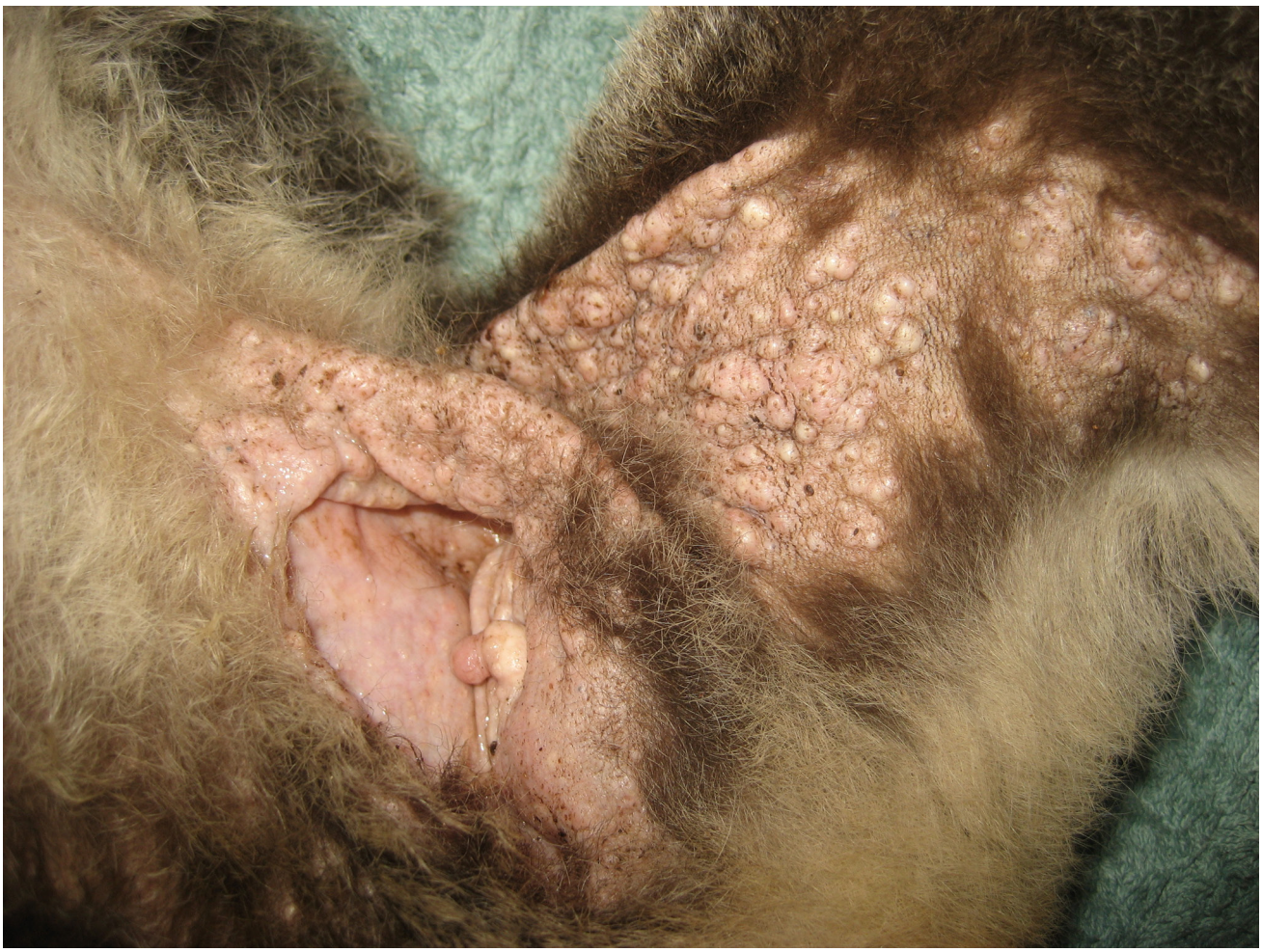

Figure 7. Sebaceous adenomata.

Immunological conditions

Koala "AIDS"

The koala "AIDS" condition remains a relatively poorly defined syndrome, characterised by chronic illthrift and a variety of clinical signs and syndromes consistent with immune incompetence, suppression or dysfunction. These clinical signs and syndromes include stomatitis (ulcerative and non-ulcerative) (Fig. 10); severe, extensive dermatitides; extensive or serious fungal infections, including cryptococcosis, candidiasis, and filamentous fungal dermatopathies; caeco-colic dysbiosis and typhlocolitis syndrome; severe, chronic chlamydiosis; severe periodontal disease, "opportunistic" and recurrent or treatment-refractive infections, and poor body and coat condition of undefined cause (Figs. 11–13). Because immune function tests are not generally available to the clinician, the diagnosis is presumptive, and based on the finding of two or more of the conditions listed above (A. Gillett, pers. comm.). These koalas may show haematologic changes consistent with immuno-suppression, such as profound lymphopaenia, but whether these findings are associated with causation, or consequent to the major disease process, is impossible to say in most cases.

Immune-mediated conditions

Thyroiditis

Interstitial, non-suppurative thyroiditis was detected incidentally in a koala that had succumbed to the koala "AIDS" condition, with concurrent chronic illthrift, typhlocolitis, and gastro-intestinal candidiasis. Histologically, there was depletion of thyroid glandular secretion and intense interstitial mononuclear inflammation, oedema and fibrosis. The cause of the inflammation was not apparent.

Plasmacytic enteritis

Plasmacytic enteritis in koalas is characterised by mild to intense lamina proprial and submucosal plasma cell infiltrates and other mononuclear cell infiltrates to a lesser degree, affecting any part of the gastro-intestinal tract from the stomach to the rectum. Many of the koalas with more severe lesions detected histologically were affected by chronic illthrift and poor body and coat condition. The plasmacyte-dominated infiltrates are reminiscent of those found in *Chlamydia*-infected organs and tissues, and it is possible that the condition is caused by chlamydial infection. At the time of writing, some of these cases were being subjected to PCR and immuno-histochemistry in an attempt to illuminate the aetiology.

Figure 8. Plantar hyperkeratosis.

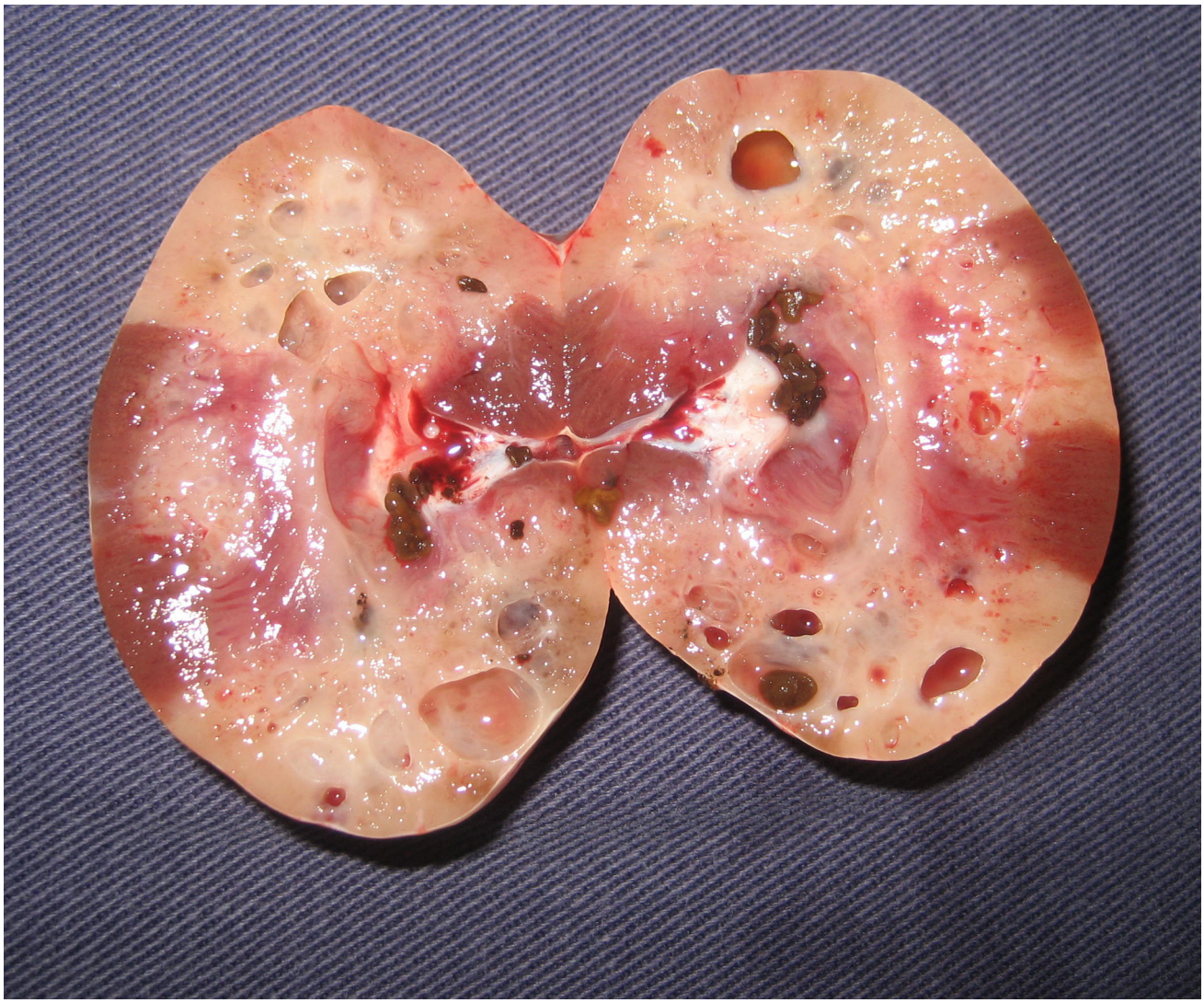

Figure 9. Polycystic kidney.

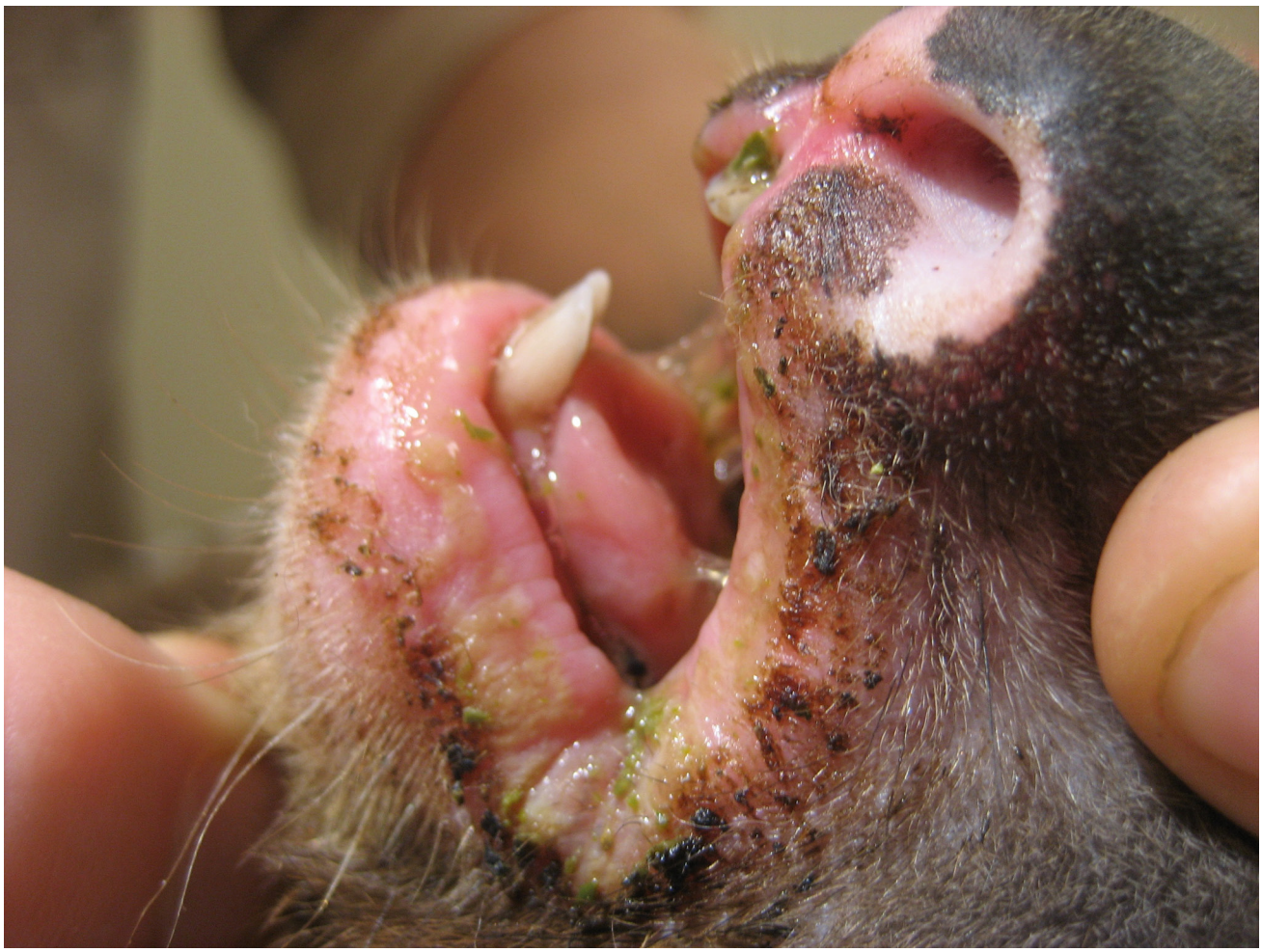

Figure 10. Stomatitis.

Figure 11. Typhlo-colitis.

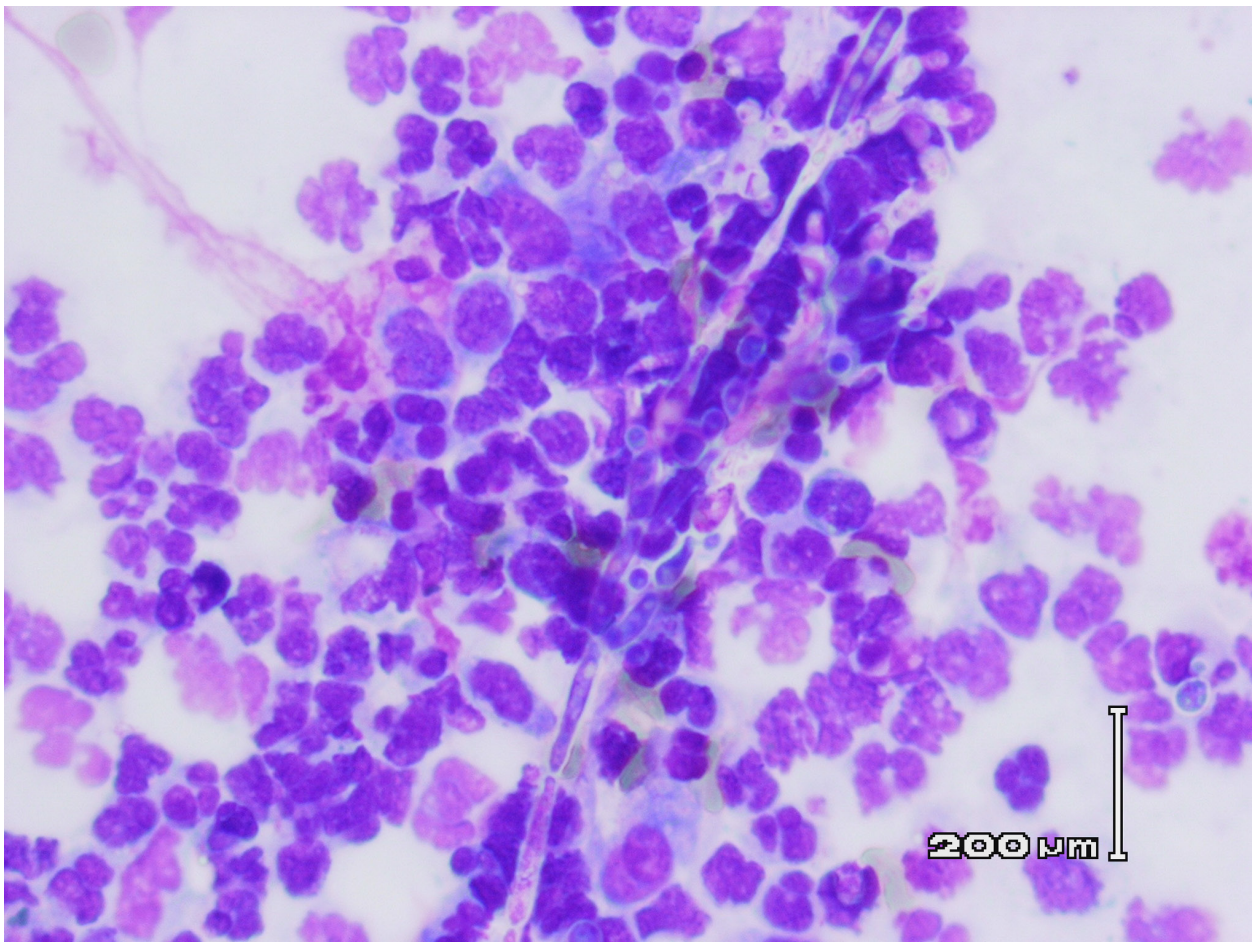

Figure 12. Candidal pseudohyphae—myocardial abscess.

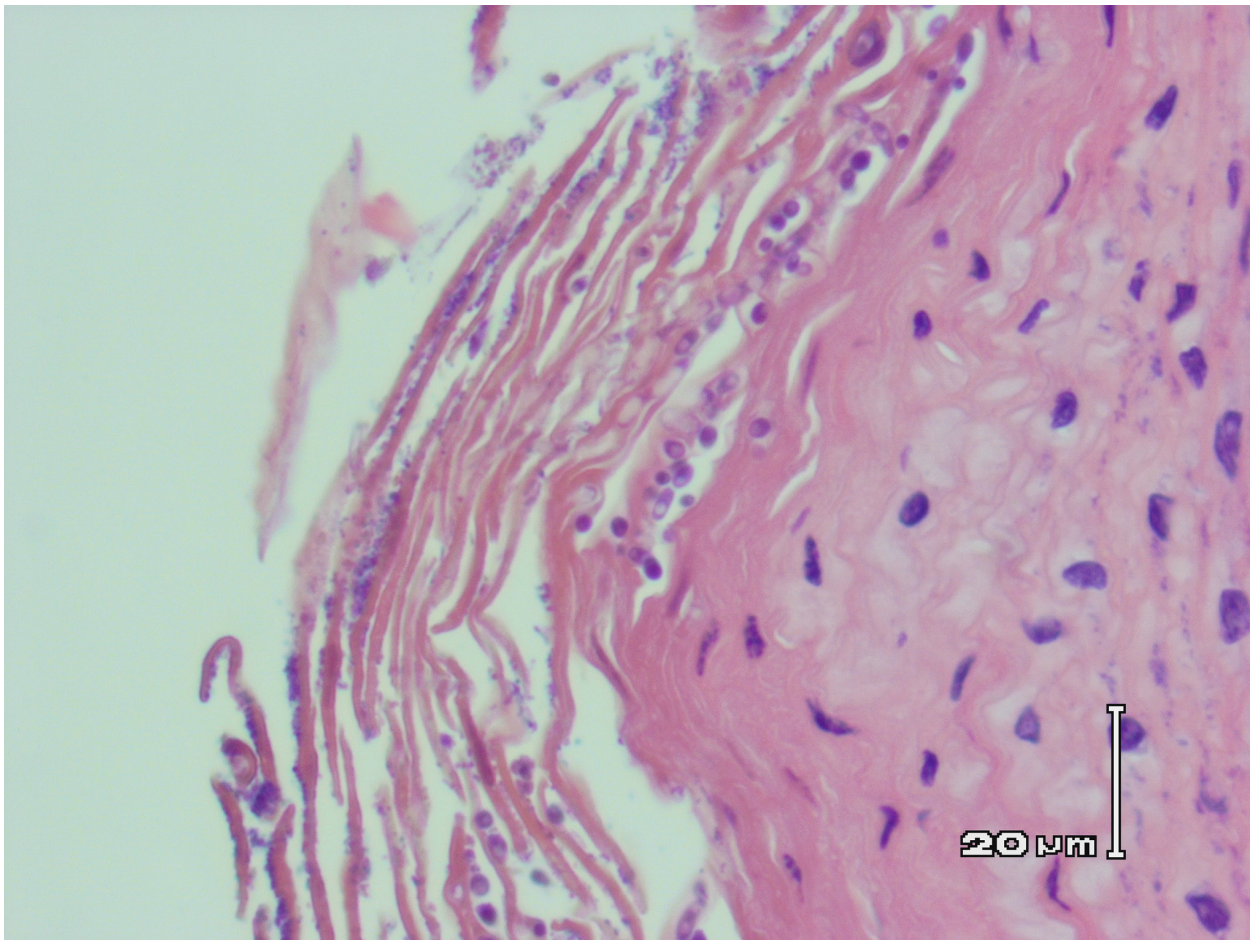

Figure 13. Candida invading oesophagus.

Chlamydiosis

While koalas with mild, self-limiting, chlamydial infections could hardly be accused of having poor or inappropriate immune responses, certainly another group of koalas—those that develop very severe, debilitating and sometimes fatal chlamydial disease—could be. In such cases, the immunological response to the infection appears to be entirely out of proportion to the inherent pathogenic risk, and consequently severe and permanent organ and tissue damage occurs because of the immune response. Whether severe, debilitating chlamydiosis is caused by highly pathogenic strains of the organism, environmental factors or KoRV-associated immune modulation or dysfunction, is a question needing urgent investigation.

Other conditions

A number of other conditions are worth considering in the case against KoRV, but are either beyond the scope of this paper to deal with, or have been adequately described by other authors. They include: pouch death of joeys; chronic illthrift (without concurrent AIDS indicators); cryptococcosis and systemic candidiasis; severe cutaneous and pox-type lesions; severe, fatal trypanosomiasis; and non-suppurative meningo-encephalitis. Undoubtedly, this list will continue to grow over time.

Prevalence and incidence of KoRV-associated disease in free-ranging koala populations

Veterinary examinations and sampling of 296 koalas from wild populations in south-east Queensland were conducted between July 2008 and March 2013. At the time of their initial examination, 23 (8%) of these koalas had disease lesions or syndromes that might be associated with the koala retrovirus. Disease lesions/syndromes found in the population included:

1 AIDS-like condition/immunodeficiency disorder (8 koalas)
2 Polycystic kidney disease (1 koala)
3 Myelodysplasia (2 koalas)
4 Neoplasia (3 koalas): 1 had renal lymphoma, 2 had leukaemia
5 Generalised sebaceous adenoma (2 koalas)
6 Severe chlamydiosis (7 koalas)

Of the 296 koalas, 126 were monitored longitudinally (for an average of 416 days per koala) and were subjected to one or more follow-up veterinary examinations (between December 2008 and March 2013). The incidence of new KoRV-associated disease lesions/illness diagnosed in the population since the initial veterinary examinations was 12.5% (in other words, each koala in the population had a 12.5% chance each year of becoming ill, or developing a new lesion). KoRV-associated disease that was detected in the population included:

1 AIDS-like syndrome/immunodeficiency disorder (8 koalas)
2 Chronic ill-thrift (unexplained poor body condition, brown, clumped coat) (5 koalas)
3 Cryptococcosis (1 koala)
4 Cancer (1 koala had a pelvic osteochondroma, 1 koala had a maxillary fibrosarcoma)
5 Severe chlamydiosis (3 koalas)

Prevalence of KoRV-associated disease—Australia Zoo Wildlife Hospital

The following table shows figures, based on 6001 koala admissions to the Australia Zoo Wildlife Hospital over the past 9 years, of possible KoRV-associated diseases. It is important to note that hospital admissions are a biased group, and figures do not necessarily represent true prevalence in free-living populations. Nevertheless, they demonstrate the relatively high rates of occurrence of these so-called KoRV-associated diseases.

A survey of wildlife veterinarians and facilities around Australia revealed that, other than dermatitis and mixed neoplasms generally in old animals, KoRV-associated disease was not observed in Victorian and South Australian koala populations, but was common in Queensland and New South Wales populations (A. Gillett, pers. comm.).

Table 1. Prevalence of KoRV-associated disease in koala admissions to the Australia Zoo Wildlife Hospital (AZWH).

AZWH category/disease	number out of 6001 cases	%
anaemia/myelodysplasia	54	0.9
myelodysplasia	42	0.7
osteochondroma	19	0.3
KoRV–AIDS	143	2.4
other Neoplasia	60	1.0
dermatitis	50	0.8
cryptococcosis	11	0.2
lymphoma (without leukaemia)	8	0.1
leukaemia	49	0.8
lymphoma + leukaemia	55	0.9
total	494	8.2

Conclusions

The genetic similarity of KoRV with other pathogenic gammaretroviruses and the occurrence of a spectrum of clinical conditions in koalas similar to those in other species is circumstantial evidence for its role in causation. However, convincing proof of its role in these diseases has not been elucidated. Further analysis of solid tumours, particularly relating to unique KoRV integration sites, and recent research efforts attempting to link particular KoRV genotypes with disease, may be productive in this regard, or at least provide further evidence for KoRV pathogenicity.

The importance of KoRV in the epidemiology and pathogenesis of chlamydial disease is still poorly understood, and therefore its role in population dynamics and local extinction is unknown. Certainly chlamydial disease has profound effects on fecundity and population persistence in some areas, but less so in other areas, with the causes of these differential impacts open to speculation. Whether KoRV has an important impact on koalas and their conservation remains an unanswered question, and certainly a topic worthy of further research efforts.

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The Origins and Ecological Impact of Koala Retrovirus

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Abstract. The genome of koala retrovirus (KoRV) has striking similarity to the gibbon ape leukemia virus (GALV) genome, suggesting the two viruses may share a common ancestor. Screening of DNA from a range of potential hosts of this putative ancestor virus revealed retroviral sequence from a grassland melomys *(Melomys burtoni)* that was closely related to sequence of both KoRV and GALV. This novel virus has been named *Melomys burtoni* retrovirus (MbRV). As grassland melomys and koalas share habitat, it is possible that there has been cross-species transmission of virus in the past.

Although a causative relationship between KoRV infection and disease in koalas is yet to be confirmed, koala populations with a high prevalence of KoRV infection have a higher incidence of diseases characteristic of retroviruses (cancer and immunosuppression) than populations with low KoRV-prevalence. Not all KoRV-infected koalas develop clinical disease. This variation in disease expression may result from differences in proviral (DNA) insertion sites among koalas, genetic variability of KoRV in different individuals or from variation in host genetics.

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Gammaretroviruses (RV) infect a large range of vertebrate hosts, and are causative agents of a number of diseases including lymphoid tumours and immunosuppression (Bendinelli *et al*., 1985; Rosenberg & Jolicoeur, 1997). Koala retrovirus (KoRV) is a relatively newly discovered retrovirus which is widespread throughout wild koala *(Phascolarctos cinereus)* populations in Australia (Hanger *et al*., 2000; Simmons *et al*., 2012). KoRV is of particular interest because it is the only known retrovirus currently undergoing a process of active endogenization in its host (Tarlinton *et al*., 2006). Koalas are known to suffer a high incidence of both chlamydiosis and cancer, and the high prevalence of KoRV has been suggested as a possible aetiological agent for immunosuppression and cancer in these animals (Tarlinton *et al*., 2008). Koala numbers in the wild have declined alarmingly since the beginning of European colonization and their geographic range has been significantly reduced. While the reasons for this decline are multi factorial, the high prevalence of KoRV and its apparent association with other diseases in koalas is a serious cause for concern.

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Origins of KoRV

Once the full sequence of KoRV was published, it was apparent that it shared striking genetic similarity with gibbon ape leukaemia virus (GALV), an exogenous, oncogenic retrovirus isolated from captive gibbons housed at the SEATO medical research facility in Bangkok, Thailand (Hanger *et al*., 2000). GALV and KoRV share such close identity that it seems likely they have a common ancestor. Since GALV was first isolated in the early 1970's there has been a degree of speculation about the source of this virus. Both GALV and KoRV are related to the murine leukaemia viruses and it has been suggested that a possible source of GALV is a related virus from a South East Asian rodent (Lieber *et al*., 1975; Callahan et *al*., 1979).

The link between KoRV and GALV adds further intrigue to this fascinating story given that a direct species jump between a primate and a marsupial that are geographically separated by several thousand kilometres seems unlikely. Following the screening of DNA from a number of potential vertebrate hosts, four partial proviral sequences from a novel retrovirus were obtained from a native Australian rodent, the grassland melomys *(Melomys burtoni)*. These sequences comprise a total of 2880 nucleotides and share remarkable identity with both KoRV and GALV. This virus has been named *Melomys burtoni* Retrovirus (MbRV) (Simmons, 2011). It shares such close identity with GALV that it could be considered another strain of GALV. Attempts to isolate infectious virus from the rodent host have so far been unsuccessful, although the provirus has open reading frames.

The grassland melomys and koala have overlapping geographic distributions throughout much of their range, and both are nocturnal (Redhead, 1983). This geographic overlap provides the opportunity for the two species to interact, and the close identity shared by KoRV and MbRV suggests there has been a cross species transmission of retrovirus between koalas and grassland melomys at some time in the past. Thus MbRV may well be the source of KoRV (or vice versa). However the genus *Melomys* does not occur in mainland South East Asia and so it seems unlikely MbRV is the direct source of GALV even though they share remarkable similarity. However *Melomys* species do occur in Papua New Guinea and it is possible MbRV may be part of a step wise transfer between several as yet unidentified species which led to the origin of the initial GALV outbreak. The discovery of GALV-related retroviral sequences in bats (Cui *et al.*, 2012) raises the possibility that these species may have been involved in this cross species transfer.

Ecological impact of KoRV infection

There is clear evidence that many retroviruses cause disease in their respective hosts. Examples include feline leukaemia virus, equine infectious anaemia, GALV and others. However the same is not true for KoRV at the present time. The link between KoRV infection and disease in koalas is at this stage more of an association rather than a demonstrated cause and effect, and although there are alarming associations between KoRV prevalence and disease in koalas more research is needed in order to clarify the role of KoRV as a pathogen. A discussion on the ecological impact of KoRV therefore needs to be addressed with this in mind.

What is clear is that wild koala populations in Australia appear to have different disease spectra depending on their KoRV status and location. Populations in the north, particularly in Queensland, but also further south, have well-documented high levels of disease whilst some southern populations, for example Kangaroo Island, are virtually free of chlamydiosis and cancer (Ladds, 2009; G. Johnsson, pers. comm. 2008). The reasons for these differences do not simply appear to be the presence or absence of KoRV and are likely more complex. Some of the possible mechanisms by which KoRV may cause disease are discussed below.

Endogenous versus exogenous

One variable which may affect the impact of KoRV is the fact that it appears to currently be in the active process of endogenization, at least in the north (Tarlinton *et al.,* 2006). When proviral copy numbers were compared between KoRV positive koalas from Queensland and Victoria the results were strikingly different. In Queensland, average proviral copy number/cell nucleus was high (165) and surprisingly uniform between animals. For Victorian koalas the number varied from about one/cell to as low as 1/10,000 cells (Simmons *et al*., 2012). Thus while KoRV appears to be endogenous in Queensland, in at least some Victorian koalas it may be present only in its exogenous form. While proviral copy number is fairly uniform in Queensland koalas, the loci where the insertions occur are variable (Tarlinton *et al.*, 2006). This may in part explain why some koalas are able to live into old age without clinical signs of disease while others succumb to chlamydiosis or cancer when relatively young. For example some koalas may have insertions in regions of their genome that impact on gene expression to the detriment of the individual's long term survivability, whereas in other koalas the proviral loci may occur in less critical regions.

In populations where the virus may be present in its exogenous form the spectrum of disease appears to be less severe. Indeed on Kangaroo Island, where the population was introduced in the 1920's and which is now highly inbred, koalas are thriving to such an extent that they are destroying their habitat.

Genetic variation

Genetic variability in both koalas and KoRV may help explain the differences in disease expression seen in different koala populations. Current research is demonstrating genetic differences in strains of KoRV isolated from different koalas and apparent differences in pathogenicity of these different strains. In addition there may well be genetic differences between koala populations which affect susceptibility to disease. For example a study of the mitochondrial control region in koalas from different populations demonstrated significant differentiation in mtDNA haplotype frequencies in these different groups (Houlden *et al*., 1999). The possible influence of such genetic variability in koala populations on the pathogenicity of KoRV remains to be investigated.

Oncogenesis

Koala retrovirus does not appear to be as oncogenic as its near relative GALV, which rapidly causes leukaemia in infected gibbons (Kawakami *et al.*, 1980). While there is an alarming incidence of cancer in koalas, it is also true that the many KoRV positive animals remain healthy. Areas with some of the highest prevalence of neoplastic disease appear to be in the north, while on Kangaroo Island cancer and chlamydiosis are rarely if ever seen (Johnnson, 2008). Whether this apparent lower incidence of disease on Kangaroo Island is due to the lower prevalence of KoRV in this population or other factors related to differences in virulence and/or genetic susceptibility of koalas remains unknown.

KoRV viraemia and disease

A study in 2005 investigated levels of KoRV viraemia and incidence of disease in 90 captive and free ranging koalas. There was a significantly higher incidence of cancer in the high viraemic group, although no significant differences between levels of viraemia and severity of chlamydiosis could be demonstrated (Tarlinton, 2005). In a later study which involved 100 wild koalas from south east Queensland, 40 animals had clinical signs of chlamydiosis and one had cancer. This study failed to demonstrate a significant link between the level of viraemia and chlamydiosis or other disease (Simmons, 2011).

Conclusions

While there are a number of serious threats to the koala's long-term survival, including habitat destruction and urban expansion, the widespread prevalence of KoRV and its association with disease in koalas is also a cause for concern. There are still large gaps in our knowledge about the pathogenesis of KoRV and variability in disease status seen in different koala populations and there is a real need for further research in this area. A potential step that could be taken to mitigate the possible negative impact of KoRV on koala viability in the shorter term could include establishment of KoRV free populations in areas of suitable habitat. Furthermore, breeding programs in the north that used KoRV animals with a family history of lack of disease susceptibility might also limit detrimental outcomes and increase survivability of these animals.

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Koala Immunology and the Koala Retrovirus (KoRV)

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Abstract. Although koala retrovirus (KoRV) is widely termed a pathogen, direct evidence for causation of disease impacts in koalas *(Phascolarctos cinereus)* remains elusive. Examination of the immune system of koalas could provide a sharper tool to investigate this but progress has been slow due to a paucity of immunological reagents in this species, and historical contradictions in research findings in this area. Our work using cross reactive antibodies to examine behaviour of resting and stimulated koala T cells (anti-human CD3); B cells (anti-human CD79b); MHCII (anti-human HLA-DP, DQ, DR) and interferon gamma (anti-bovine IFNg) by flow cytometry have revealed some features consistent with a skew to a Th2 (B cell) immune focus. Assessing the role of KoRV in immunomodulation in koalas clearly requires more in-depth research. We have used recent advances in genomics of other marsupials to develop tools necessary to assess KoRV's effects on koala immune function in free-ranging, captive and in-vitro systems.

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Immunosuppression in koalas: is it clear-cut?

Early researchers working on koala immunology formed the belief that koalas were immunologically "lazy", and this has coloured perceptions of koala immunology in the broader community ever since. This idea was originally put forward based on apparently slow seroconversion to chlamydial infection, and a limited cellular response to overwhelming sarcoptic mange in a small number of koalas (Brown, 1988). It sparked a series of studies that pioneered marsupial immunology but also set the scene for two decades of intriguingly disparate findings: lymphoid tissues of koalas are generally more sparsely populated than those of many species (Wilkinson *et al*., 1992a), yet the arrangement of these tissues is consistent with those of eutheria, with similar distribution of T and B cells (Hemsley *et al.,* 1995, 1996a, b); initial experiments indicated slow and weak local cutaneous delayed type hypersensitivity reactions (Wilkinson *et al*., 1994), yet koalas are clearly capable of mounting prolific lymphoplasmacytic responses, with their inflammatory infiltrates and distribution of B and T lymphocytes in chlamydial disease being very similar to the non-protective, deleterious response to conserved chlamydial heat shock proteins that induces pelvic inflammatory disease in humans (Hemsley & Canfield, 1997; Morrison, 1991). Similarly, in contrast to poor antibody responses described initially (Wilkinson *et al*., 1992b; Wilkinson *et al*., 1994), recent vaccine trials induced strong humoral and cellular responses (Carey *et al*., 2010; Kollipara *et al*., 2012). Also, in response to natural *Chlamydia pecorum* infection, koalas develop neutralizing anti-MOMP antibodies (Girges *et al*., 1993), and also develop high anti-hsp60 and hsp10 antibody titres in association with chlamydial reproductive tract fibrosis (Higgins *et al*., 2005), as do women similarly affected by *C. trachomatis* (Domeika *et al*., 1998; LaVerda *et al*., 2000).

Clearly, we have evidence of outcomes of a functional adaptive immune response in the koala. However, in terms of its strengths and weaknesses, and the evolutionary forces that have shaped it, we are just beginning to scrape the surface. KoRV as a potential immunosuppressive agent needs to be considered in the context of a range of forces

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and trade-offs shaping a co-evolutionary relationship between a host, its environment and a range of potential pathogens. Many diseases of koalas are shared across other marsupials, as might be expected, based on the shared environments in which they evolved and now inhabit; *Cryptococcus gatti* invasion occurs in immunocompetent hosts of many species (Krockenberger *et al*., 2003); a range of marsupial species show tendency to disseminated mycobacterial infection (as do badgers and ferrets) (Buddle & Young, 2000); and wombats are also highly susceptible to sarcoptic mange (Skerratt, 2005). At the same time, it would not be surprising to find species- or even habitat-specific weaknesses or strengths in the koala immune response. In theory, if *Chlamydia pecorum* were a European introduction, a relatively non-gregarious species on a lean energy and nutrient budget, such as the koala, might (or might not) have evolved a more limited investment in adaptive immunity than that needed to protect against contagious pathogens in a more social species. As another hypothetical example on a finer scale, those koala populations needing serological defence against the paralysis tick *(Ixodes holocyclus)* toxin (i.e. in coastal regions of Queensland and New South Wales, where chlamydial disease is also generally most common) might benefit from a strong antibody-based (Th2) immunity, in a trade-off, at the expense of the cellular (Th1) response considered critical to elimination of *Chlamydia*. When laid over the impact of a history of hunting, regional translocation and habitat fragmentation on immune gene (MHCII) diversity (Lau *et al*., 2013, 2014); the likelihood of some MHCII variants being associated with survival and chlamydial disease (Lau, 2013); and diversity of chlamydial strains among and within koala populations (Higgins *et al*., 2012; Marsh *et al*., 2011); it becomes evident that we are dealing with a complex system. This highlights the need for integrated studies including both eco-immunological and epidemiological studies in free-ranging animals in a variety of populations and disease states, and exploration of pathogenic mechanisms in more controllable captive or *in vitro* systems.

Is KoRV immunomodulatory in koalas? Do we have enough data?

Associations with infectious disease are equivocal (Simmons, 2011; Tarlinton *et al*., 2005), though this might be due to the multi-factorial nature of disease, especially in populations of free-ranging animals. The most direct evidence for immunomodulatory effects of KoRV comes from the effect of purified KoRV on cytokine expression by cultured human PBMCs, whereby it induced elevated expression of interleukin-6 (IL-6), IL-10, growth-related oncogene (GRO) and monocyte chemotactic protein-1 (MCP-1) (Fiebig *et al*., 2006). This is consistent with the highly conserved nature of the retroviral transmembrane envelope protein p15E, or immunosuppressive domain (ISD), across KoRV, GALV, MuLV and FeLV (Fiebig *et al*., 2006); and the wide range of its effects on cells of other species in vitro, including: inhibition of respiratory burst and chemotaxis of the human monocyte; inhibition of macrophage accumulation at inflammatory foci in mice; suppression of neutrophil function; inhibition of human natural killer cell activity; inhibition of lymphocyte proliferation and mitogenic cytokine production; and increased production of interleukin-10 (IL-10) (Denner, 1998). Whether these effects occur in koalas and whether these have significant downstream effects in this species has not yet been tested.

Due to constant pathogen-driven selection, immune molecules are among the least conserved between species. Our ability to examine koala immune profiles has, therefore, been limited to detection of antibodies, lymphocyte proliferation assays, immunophenotyping by flow cytometry with a limited number of cross-reactive antibodies to conserved (mostly intra-cytoplasmic) domains (T cell, anti-human CD3; B cell, anti-human CD79b; MHCII, anti-human HLA-DP, DQ, DR; IFNg) (Higgins *et al*., 2004; Lau *et al*., 2012) and, very recently, qPCR for IL10, IFNg and TNFa (Mathew *et al*., 2013a; 2013b). Our recent immunophenotypic studies on captive, KoRV-positive koalas (Lau *et al*., 2012) revealed some interesting features: elevated numbers of B cells relative to other species (1.0–4.9×10^6 cells/ml vs 0.17–0.56×10^6 cells/ml, respectively), and absence of MHCII expression on stimulated and non-stimulated T cells. Both Concavalin A (ConA) and Pokeweed Mitogen (PWM) induced MHCII up-regulation in koala B cells but not T cells; in contrast to the marked (e.g., 50–90%) MHCII expression on T cells of all other species studied to date, but mice (Byrne *et al*., 2000; Rideout *et al*., 1992; Schwartz *et al*., 2005; Holling *et al*., 2004). Ability of koala T cells to respond to mitogens was evident, in that PWM induced proliferation of T and B cells and ConA induced preferential proliferation of T cells in our study and, in our previous study, 14% of PMA-Ionomicin stimulated koala lymphocytes labelled strongly with cross-reactive anti-IFNg antibodies (Higgins *et al*., 2004). Increased B cell numbers and absence of T cell MHCII expression would be consistent with retrovirus-associated increased Th2 profile (Denner, 1998; Haraguchi, 2008). However, it could alternatively reflect an evolutionary adaptation within the koala's immune response and this phenomenon needs to be examined in more detail KoRV positive and negative koalas from a range of habitats and disease states.

Where to now: testing the effects of KoRV on koala immune function

By using available sequence from non-koala marsupial genomes (common opossum *Monodelphis domestica*, tammar wallaby *Macropus eugenii*, Tasmanian devil *Sarcophilus harrisii*, common brushtail possum *Trichosurus vulpecula*) (Morris *et al*., 2010) we have recently generated koala sequence and developed and validated a series of koala-specific qPCRs for immune genes CD4, CD8, IL-10, IL-4, IFNg, IL-6 and several reference genes (Maher *et al.,* 2014). We are applying these to our collections of samples from KoRV positive and negative free-ranging koalas, and cells of captive koalas in *in vitro* studies to better describe normal and abnormal immune function in these koalas.

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An Examination of Disease in Captive Australian Koalas *(Phascolarctos cinereus)* and Potential Links to Koala Retrovirus (KoRV)

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Abstract. Koalas *(Phascolarctos cinereus)* are known to suffer from a range of neoplastic and immunodeficiency-related disorders but the importance of these conditions to captive koala populations has not previously been thoroughly examined. This study aimed to improve our understanding of disease in captive koalas by conducting a detailed questionnaire survey across most facilities that house koalas in Australia. Responses were received from 16 facilities across five Australian states that resulted in disease information for a total of 264 koalas. The collated data indicated that neoplasia is the major type of diagnosed disease affecting captive koalas, with lymphoma clearly the most common (c. 40%). A variety of other disorders were reported including bone marrow disease (especially leukaemia), cryptococcosis and dermatitis, the latter of which was the only condition reported from all five states. These data suggest a higher incidence of disease in facilities in Queensland and New South Wales, which are predominantly comprised of northern koalas. Mortality records spanning up to 28 years were received from six of the surveyed facilities which indicated that of 303 deceased captive koalas, 32% of deaths were attributable to the diseases mentioned above. It is likely that the prevalence of disease reported here is an underestimate due to the lack of, or inconsistent application of, appropriate diagnostic investigations amongst facilities from all states. Given that previous research suggests that northern koalas are ubiquitously infected with koala retrovirus (KoRV) and that they have higher viraemic loads than their southern counterparts, there may be a link between KoRV and the higher disease expression among northern koalas postulated here. Further research is required to determine if there is a causal link between KoRV and the predominant diseases among captive koalas reported in this study.

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Wild koala *(Phascolarctos cinereus)* populations are found across a broad geographic range in eastern and south-eastern Australia and occur in the states Queensland (QLD), New South Wales (NSW), Australian Capital Territory (ACT), Victoria (VIC) and South Australia (SA) (Martin & Handasyde, 1999). Koalas are generally referred to as either "northern" or "southern", a description which is largely determined by state borders. Northern koalas are distributed from north QLD to south of the NSW/VIC border and southern koalas are distributed through most of VIC and SA (Carrick, 2013). Variations in appearance between northern and southern koalas are evident and most notably include longer fur length and larger size in southern compared to northern koalas. In many parts of the species range, populations of wild koalas are declining at alarming rates and local extinctions have already occurred across considerable areas. Declines are largely attributed to habitat loss, trauma (road and domestic/wild dog attacks) and disease within populations (principally chlamydiosis). A further potential threat to koalas that is receiving increased scientific attention

is koala retrovirus (KoRV). Koala retrovirus (KoRV) has become endogenized in koalas and appears to be 100% prevalent in wild (and captive) QLD and NSW koalas and its presence is being increasingly identified amongst wild koalas in the southern states of Australia VIC and SA (Ávila-Arcos *et al*., 2013). Retroviruses are known to affect many vertebrate species and can lead to immunosuppression and in some cases neoplasia such as leukaemia and lymphoma (Rosenberg, 2011). These conditions are prevalent in wild and captive koalas and there is speculation that KoRV may play a role in inducing neoplastic and immunosuppressive disorders in koalas.

While KoRV appears to be widespread in koalas, the circulating viral load may be of greatest importance in understanding the virus' impacts in koalas. An association appears to be evident between the presence of high circulating levels of KoRV and immunodeficiency and neoplastic disorders in both wild and captive koala populations throughout Australia (Hanger *et al*., 2000; Tarlinton *et al*., 2005). Further research is currently being conducted to better understand the role of KoRV and virus load upon infected individuals, and the potential management implications for captive animals.

The most recent tally of captive koalas in Australian facilities from the Zoo and Aquarium Association (ZAA) identified 514 northern koalas and 112 southern koalas currently being held across six of Australia's seven states. All northern animals, except four, are held in QLD, NSW and WA facilities and almost 80% of the southern animals are listed in VIC and SA. Captive populations originated from wild gene pools of both southern and northern koalas but now captive breeding for zoological collections is commonplace throughout Australia. Captive koalas are also routinely moved between Australian zoos and even exported from Australia to international zoological institutions. Occasionally, wild animals are also incorporated (through government-approved species management programs) into established captive colonies within Australia to use as display animals or in captive breeding programs. Wild koalas approved for such placements have often sustained injuries deeming them not fit to return to the wild, are orphaned hand-raised individuals that have not demonstrated wild instincts or are infertile due to *Chlamydia*-related reproductive disease. In the latter case, certain state governments prohibit the re-release of infertile koalas to the wild so after chlamydial treatment and ovariohysterectomy, to remove the diseased reproductive tract, these koalas can be placed in a captive management situation for display and education purposes.

Across the facilities that house captive koalas there are increasing reports of neoplastic and potential immuno-deficiency disorders. Previous investigations of some captive populations have identified as many as 55% of deaths were attributable to lymphoid neoplasia (Hanger, 1999). Lesions appear to be more frequently reported in the northern koalas and include leukaemia, myelodysplasia, tumours (Fig. 1) and immunodeficiency-like syndromes that tend to include generalized dermatitis and/or stomatitis/oral ulceration (Fig. 2). Anecdotal reports of recognized patterns in the above syndromes from one generation of captive koala to the next have also been observed, with some facilities having seen more than three generations succumb to the same form of neoplastic diseases (leukaemia and lymphoma) at similar ages (M. Panayiotou, pers. comm.). Other conditions such as cryptococcosis are also encountered in captive populations as an opportunistic infection likely related to immunosuppression (Hanger *et al*., 2003) and overwhelming environmental load of the organism (Krockenberger *et al*., 2002).

To date, very limited information has been available on the prevalence of specific neoplastic and immunodeficiency-like syndromes in captive koalas throughout Australia. The aim of this manuscript was to collate detailed information on the prevalence of specific neoplastic and potential immunosuppressive disorders within these facilities, examine currently employed diagnostic techniques and discuss the potential role of KoRV in the expression of identified diseases.

Materials and methods

A questionnaire survey was developed to elicit detailed information from relevant captive koala facilities on the type and prevalence of neoplastic and potential immunosuppression-related syndromes in captive koalas. The survey was electronically distributed to veterinary and wildlife network email server lists including the Australian Wildlife Health Network (AWHN), Wildlife Disease Association (WDA) Australasian section and ZAA (Australasian section). As such, the survey should have been received by representatives of all 39 Australian facilities that house captive koalas and are members of the ZAA. The survey was also directly emailed to individual veterinarians, curators and other wildlife facilities known to the author. The audience captured by this approach was in the order of 750 people/facilities (500 AWHN, 158 WDA, 82 ZAA and 10 personal contacts).

Questionnaire recipients were asked a series of questions regarding captive koalas at their facility. Firstly, they were asked the total number of captive koalas currently at their facility and if they had acquired wild koalas for display or breeding purposes. Participants were then asked to state the number of koalas affected by leukaemia, myelodysplasia, erythroid dysplasia, chronic dermatitis or other signs of immunosuppression (including chronic ill thrift, recurrent or persistent stomatitis, oral ulceration and severe debilitating chlamydiosis), cryptococcosis, osteochondroma, lymphoma or other neoplasia. For ease of reporting in this manuscript, leukaemia, myelodysplasia and erythroid dysplasia have been categorized as "bone marrow conditions", dermatitis, stomatitis and signs of immunosuppression are categorized as "AIDS-like conditions", cryptococcosis remained as "cryptococcosis" and osteochondroma, lymphoma, and other neoplasia are categorized as "tumours".

Finally, participants were asked if they had recognized any of the mentioned syndromes in multiple generations of the same genetic lineage, and what procedure/s they routinely perform when examining an koala ill from an unspecified cause (survey options included blood smear, bone marrow, abdominocentesis or none at all).

Once all data regarding the diseases of interest was collated from returned surveys an additional information request was sent to each of the 16 facilities. The requested information included a tally of total koala numbers housed during the history of their facility, the time frame covering the period of provided records, and mortality records during the reported time frame. Mortality records were requested for all of the disease conditions listed in the initial survey, defined as diseases of interest (DOI), as well as for mortalities due to other causes, so that the proportional contribution of disease-related deaths could be calculated.

All returned surveys were received electronically via email. Participants were not asked within the survey to define what facility they were from, however this

Figure 1. Basal cell carcinoma on the face of adult 10 year old male northern koala *(Phascolarctos cinereus)*.

information was obtained once surveys were returned in order to separate findings by state. Participants were not asked to separate koalas into numbers of northern versus southern koalas in their facility.

Results

The total number of facilities/individuals that responded to the initial survey was 16. Information from one facility was provided via the Australian Registry of Wildlife Health (ARWH) and not the facility itself, whilst 15 facilities provided direct responses. Six participants were from NSW, five from QLD two from VIC, two from SA and one from Western Australia (WA). The total number of koalas presently held in the participating facilities totalled 414 with 282 in QLD, 52 in NSW, 46 in SA, 24 in WA and 10 in VIC.

Disease information from the initial survey was provided for a total of 264 koalas, comprising 189 from QLD (71.5%), 64 from NSW (24.3%), six from WA (2.3%), three from VIC (1.1%), two from SA (0.8%). Tumours were overwhelmingly the most prevalent condition noted by participants (56% of 264 koalas). Within this category lymphoma was the most common diagnosis (Fig. 3), followed by "other neoplasia", and almost all tumours were in QLD and NSW animals (Fig. 4). Seventeen "other tumours" were reported in the "other neoplasia" category and are listed in table 1. Some conditions listed under "other tumours" were not specifically identified by tumour type, including ovarian cancer, papilloma and unspecified neoplasia, but these were included in the final dataset. In QLD and NSW, which in combination accounted for more than 95% of disease cases examined here, lymphoma was clearly the most prevalent tumour representing approximately 40% of all diseases in these states (Fig. 5).

AIDS-like conditions appeared to account for the next most prevalent disease processes (20% of 264 koalas), with most facilities noting dermatitis as a common finding. The percentage for dermatitis represented in Fig. 3 is an underrepresentation because one facility commented that they saw "lots" of animals with this condition, but they did not assign a numerical value.

Bone marrow conditions represented the third most prevalent category (14% of 264 koalas). However, cases of leukaemia represented the vast majority of reported diseases in this category, being more than five times more common than other reported forms of bone marrow disease (Fig. 3). Virtually all cases of bone marrow disease were diagnosed from QLD and NSW facilities (Fig. 5). Cryptococcosis was noted in 29 (11%) cases.

Two facilities noted a potential hereditary pattern of disease (specifically lymphoma and lymphoma with leukaemia). Both facilities were from Queensland and housed majority northern subspecies of koalas. The remaining facilities reported that no pattern was noted and a response was not received from one facility as the information about its koalas was obtained through the AWHN archive and not the facility itself.

Fifteen facilities provided information regarding which procedures they routinely performed when examining a

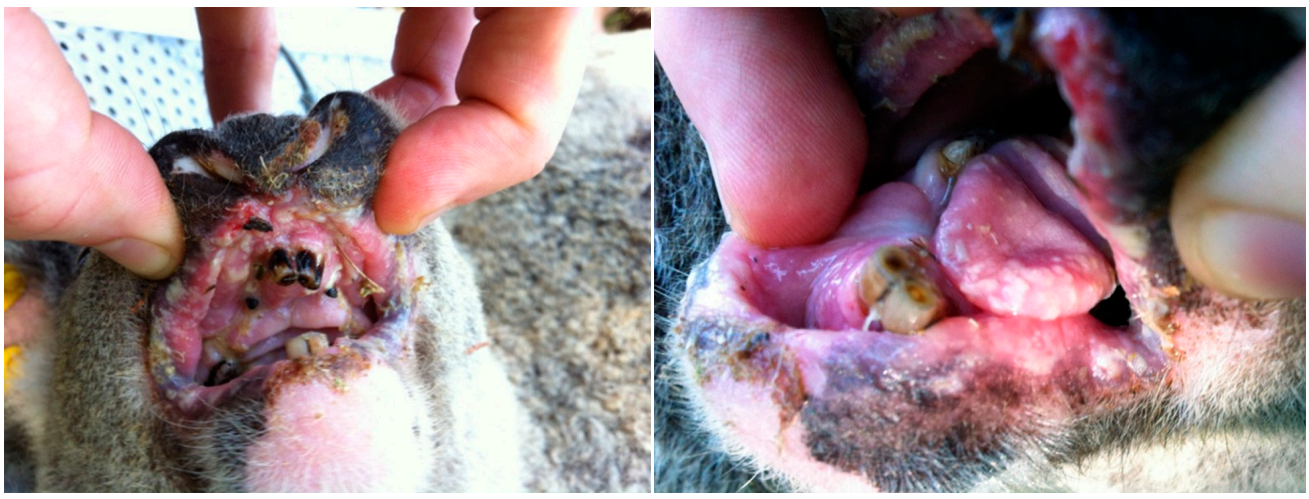

Figure 2. Stomatitis and oral ulceration in an adult (exact age unknown) female northern koala *(Phascolarctos cinereus)*.

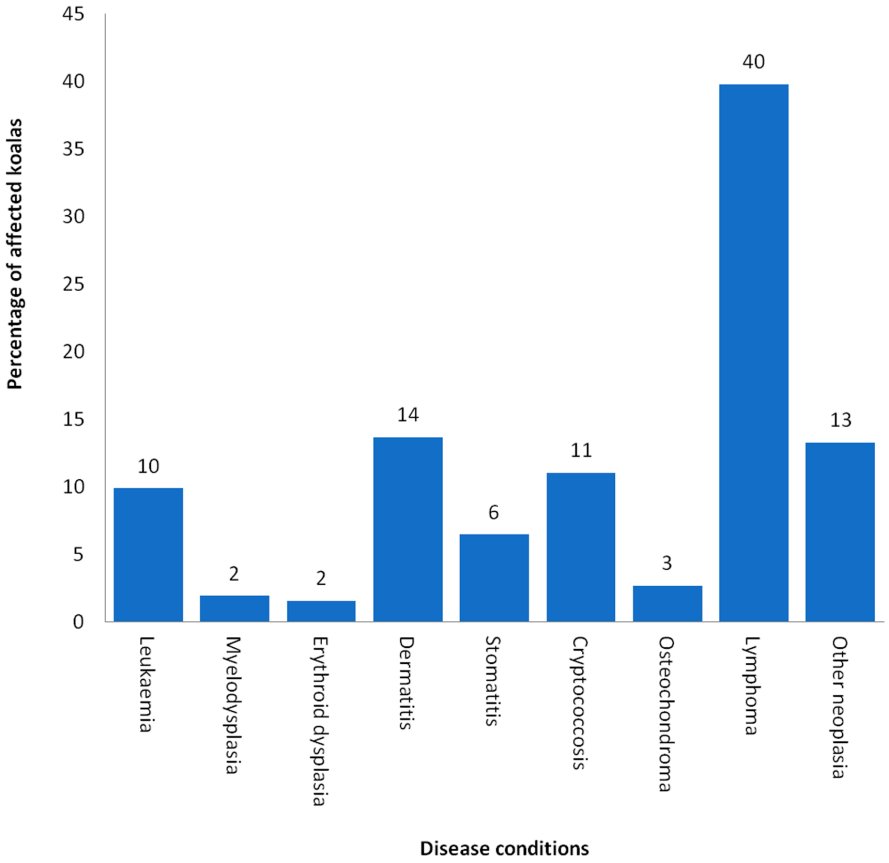

Figure 3. Percentage of captive koalas *(Phascolarctos cinereus)* (n=264) affected by conditions reported from 16 surveyed facilities in Australia.

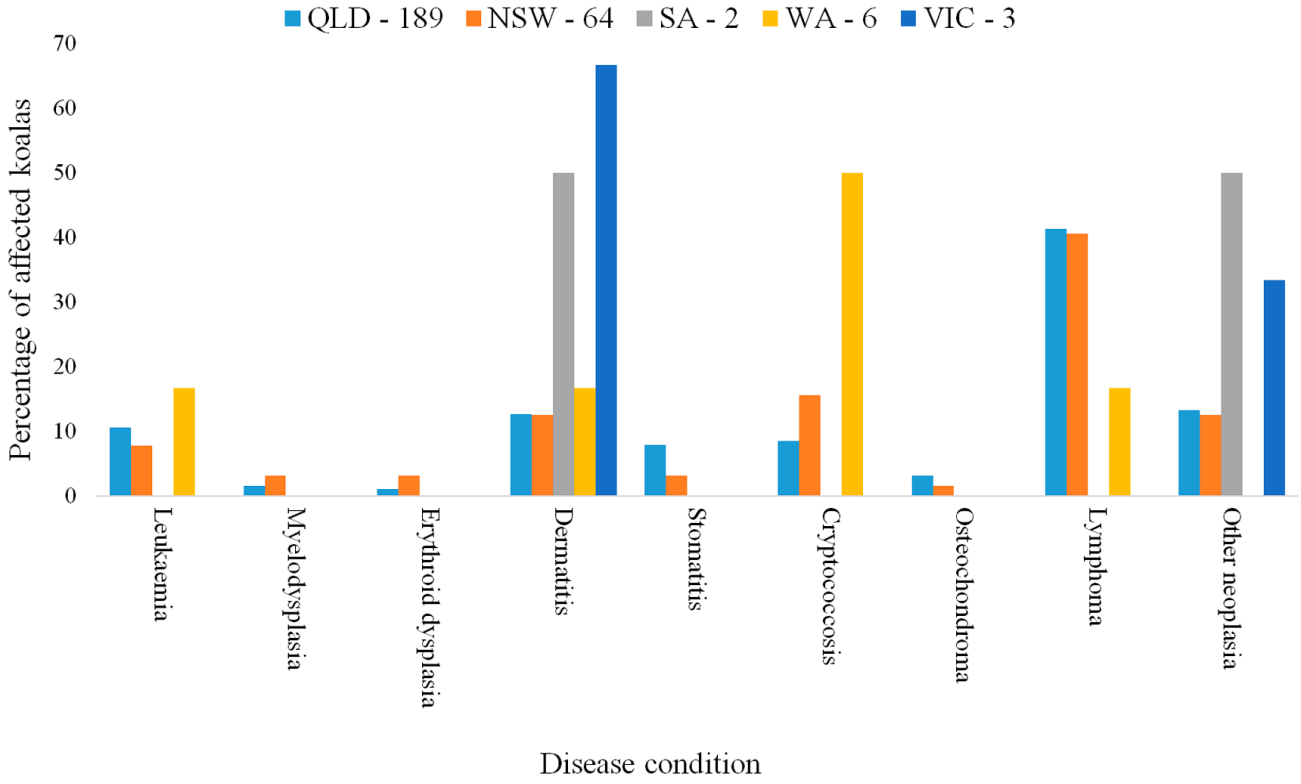

Figure 4. Percentage of disease in 264 captive koalas *(Phascolarctos cinereus)* identified by Australian state.

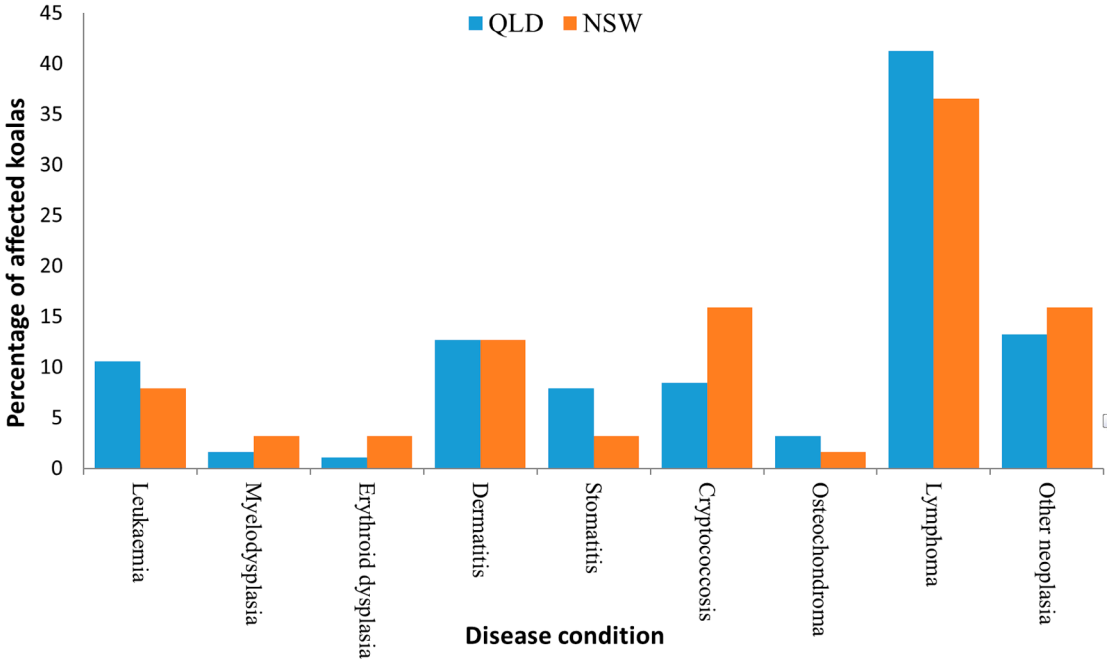

Figure 5. Comparison of disease prevalence in captive koalas between QLD (n=189) and NSW (n=64) as reported from 11/16 facilities (QLD 5, NSW 6).

koala ill from unspecified cases. One facility reported that they did not routinely conduct any diagnostic procedures. Of the remaining 14 facilities, all but one routinely conducted blood smears; two facilities stated they routinely utilized one additional diagnostic technique (bone marrow aspirate or abdominocentesis), whilst only two facilities routinely conducted all three procedures.

Responses to the additional information request on koala mortalities were obtained from six facilities across three states (QLD 2, NSW 3 and WA 1). The period of time reported on by each facility varied from five years (NSW) to 28 years (WA) with four of the six facilities able to provide data for at least a 10 year period. The total number of koalas housed collectively amongst the facilities for the time frames outlined was 533 with mortality records available for 303 animals. When analysed as a combined dataset, 32% of these mortalities were attributed to the DOI whilst the remaining 68% consisted of deaths due to other causes (including pouch mortality, gastrointestinal conditions, septicaemia and age related death). Of the DOI the most common causes of death were lymphoma (53%), other neoplasia (15%) and leukaemia (11%).

When examined on a state basis 192 mortality records were available from QLD, 84 from NSW and 27 from WA. When expressed as a percentage, mortality rates due to the DOI were similar between QLD and NSW (35% and 32% respectively) but considerably lower from WA (7%) (Fig. 6).

Table 1. List of different types of neoplasia identified under "other neoplasia".

description	count
cholangiocellular carcinoma	1
fibrosarcoma	2
haemangiosarcoma	3
intestinal adenocarcinoma	1
leiomyoma	1
mesothelioma	7
myxosarcoma	1
nephroblastoma	1
osteosarcoma	4
ovarian cancer (type not described)	1
papilloma	1
renal cystadenocarcinoma	1
periosseous giant cell tumor	1
phaeochromocytoma	1
sarcoma	1
squamous cell carcinoma	3
other—unspecified	4
tally	34

Discussion

Although neoplasia has been well documented in wild and captive koalas, this study provides the first comprehensive examination of the types and prevalence of neoplastic and potential immunodeficiency based disease in captive koalas throughout Australia. The results of the study indicate that tumours, particularly lymphoma, are the main form of disease identified in the sampled captive populations, which is similar to what previous studies have found in wild and captive koalas (Canfield, 1990; Canfield *et al*., 1990; Connolly *et al*., 1998; Hanger, 1999). The study has also shown a high number of other neoplasias occurring in captive koalas (see Table 1).

Dermatitis was the only reported condition in common between all five examined states, although it represented only a relatively small proportion of all disease (14%). This condition alone is generally non-fatal but may be indicative of underlying immunosuppression. Interestingly, lymphoma, osteochondroma, cryptococcosis, stomatitis and all forms of bone marrow disease were only reported from QLD, NSW and WA.

The vast majority of data collated in this study was derived from koalas in QLD and NSW facilities. Interestingly, these data show the relative prevalence of each disease is very similar across these two states, as highlighted in Figure 5.

Collectively, historical mortality rates due to the diseases of interest amongst the two QLD and three NSW facilities were similar, despite QLD providing more than double the number of koala mortality records than NSW, 192 and 84

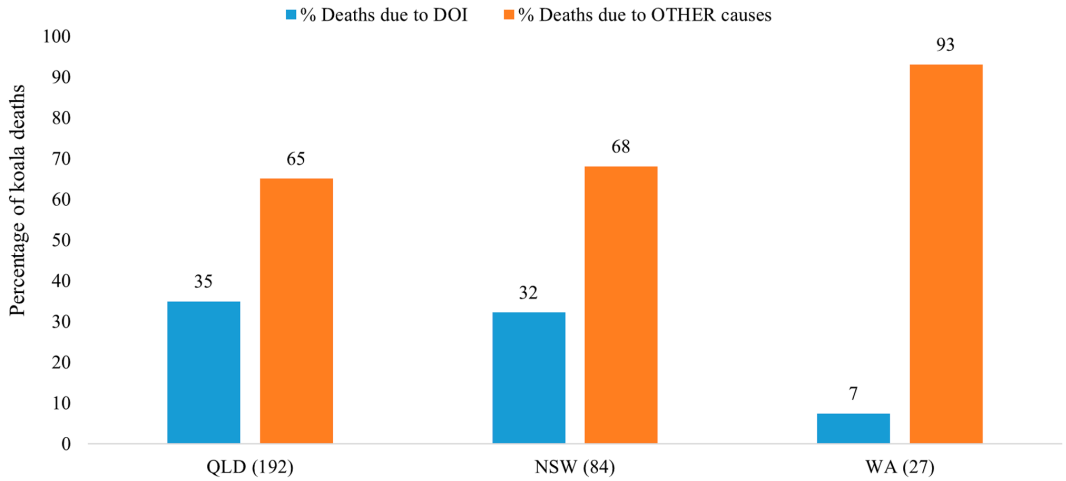

Figure 6. Comparison of causes of mortality amongst captive koalas in QLD (2 facilities), NSW (3 facilities) and WA (1 facility) for up to a 28 year reporting period.

respectively. Mortality rates due to the diseases of interest could be much higher than that represented here for QLD as unfortunately two of the four largest koala facilities were unable to provide the requested mortality record information. Of the QLD facilities that did respond, all housed a majority of northern koala species with reporting periods of up to 15 years. The maximum reporting period for NSW facilities was also 15 years whilst WA provided 28 years of mortality data and both states housed predominantly northern koalas. When all six facilities were analyzed collectively the mortality rate of captive koalas attributed to the diseases of interest in this manuscript was in the order of 32% with lymphoma identified as the most common disease resulting in death (53%). This is relatively consistent with findings in North America and Europe (G. Pye, pers. comm.).

No mortality records were available for the examined facilities from SA and VIC. However, although there are currently more than 50 captive koalas held in these states and there are likely to have been considerably more than this in the history of the facilities, only five cases of disease were reported from these states in this study. This small number of disease cases makes it difficult to draw any meaningful conclusions about the main cause of disease or the level of mortality due to disease, but it does suggest a low level of disease amongst captive koalas in SA and VIC, which are almost exclusively southern koalas. It has previously been suggested that southern koalas suffer less from all forms of disease (including chlamydiosis) than their northern (QLD, NSW) counterparts.(Simmons *et al*., 2011).

It has been suggested that KoRV may directly induce neoplastic or immunosuppressive disease in koalas, especially among those individuals with high viral loads (Tarlinton *et al.*, 2005). Recent research into the prevalence of KoRV across the distribution of wild koalas revealed that all examined koalas from QLD (n = 277) and NSW (n = 100) tested positive for KoRV as identified by Hanger *et al*. (2000) and that proviral copy number was much higher in these animals than southern counterparts (Simmons *et al*., 2011). Although thorough sampling for KoRV has not been conducted on captive koalas in Australia, it is reasonable to presume that captive koalas bred from wild QLD or NSW koala lines will have similar levels of viraemia and proviral copy number. Therefore, it is possible that the presumed higher incidence of disease among northern koalas in this study may infer a relationship between high levels of KoRV and the prevalence of disease.

Queensland was the only state where a potential hereditary pattern of disease was reported. Two facilities, both housing northern koalas, reported successive generations dying from the same disease, namely lymphoma or leukaemia. One facility noted these conditions occurring in at least three generations of koalas, with all animals succumbing to disease at similar ages, suggesting there may be a heritable susceptibility to disease expression.

This study has identified what disease conditions are present in captive populations and what diagnostic tools are routinely used at the examined facilities. It is suggested that the prevalence of all the listed conditions of interest may be underestimated for a number of reasons. Firstly, the presence of bone marrow diseases can only be definitively diagnosed if bone marrow is assessed. This can be done antemortem or post-mortem but requires a core marrow sample to be examined cytologically or histopathologically. Only three facilities (18%) reported using a bone marrow sampling technique when assessing ill koalas. This does not exclude the use of histopathology for diagnosis post-death but does indicate that antemortem techniques for bone marrow assessment are infrequently used by most facilities. Also, bone marrow may be less commonly sampled at necropsy unless there is suspicion of bone marrow disease. For example, in an animal that is aged or that may not have significant and obvious abnormalities in their blood film consistent with marrow disease, sampling of bone marrow may not be routinely done.

Diagnostic investigation may also be constrained where veterinary and laboratory assistance is extremely limited (for example, due to financial constraints and location/access difficulties). This may be especially true of smaller facilities. Therefore, it is highly plausible that many conditions may be missed or under diagnosed due to the lack of thorough diagnostic investigation and post mortem examination.

Further information regarding the prevalence of disease in captive koala populations can only be gained by the development of, and adherence to standardized diagnostic investigation and post mortem techniques in the future. This, in conjunction with routine screening of animals for KoRV and determination of viraemic loads may improve our knowledge on whether animals with high viraemic loads, more commonly develop neoplastic and immunosuppressive syndromes. Given the reports from two facilities of potential hereditary patterns of neoplastic disease this area needs further investigation. As KoRV is

an endogenized virus, accumulated amplification of virus through generations is a potential concern. If KoRV is found to definitively induce neoplasia then it is plausible that increasing reports of neoplasia in captive animals from the same genetic lineage will occur. This may be a much greater issue in the management of captive of koalas than in wild populations. If standardized diagnostic techniques, record keeping and necropsy protocols can be implemented throughout all institutions that house koalas then more accurate information can be gathered on the real prevalence of neoplastic and potential immunosuppressive disorders, and their relationship with KoRV. With improved knowledge in this area perhaps the gap between KoRV as a causative factor in these diseases can be closed and strategies essential to the future management of captive koala populations can be implemented.

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Molecular Characterization of Koala Retroviruses Isolated from Koalas *(Phascolarctos cinereus)* Reared in Japanese Zoos

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Abstract. In northern Australia most koalas *(Phascolarctos cinereus)* are infected with the gammaretrovirus known as koala retrovirus (KoRV). KoRV is believed to be currently endogenizing into its host. Koalas were first introduced into three Japanese zoos in 1984 and now about 50 koalas are held in eight zoos. In 2007 KoRV was isolated from koalas reared in Japanese zoos, and, for the first time, an infectious molecular clone termed pKoRV522 was constructed. Using the molecular clone and KoRV isolates, we revealed the budding mechanism of KoRV and genomic diversity of KoRVs isolated from Japanese koalas. We found that KoRV utilizes the multivesicular body-sorting pathway. We also discovered a novel KoRV subgroup, named KoRV-J, which utilizes thiamine transport protein 1 as an entry receptor. The original KoRV, which utilizes Pit-1 as an entry receptor, is now named KoRV-A. In two Queensland koalas examined, the copy numbers of KoRV-J was less than 1 copy per cell and varied in tissues. These data, at least in these two koalas, suggest that KoRV-J is an exogenous retrovirus not an endogenous retrovirus.

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Endogenous retroviruses (ERVs), occupy about 8 to 13 percent of mammalian genomes. Most ERVs are defective due to genomic mutations and deletions. However, some ERVs retain functionality and contribute to host physiological processes, exemplified by the human syncytins in placentation (Feschotte & Gilberet, 2012). In this regard, ERVs are believed to play a role in the evolution of mammals, yet the process of endogenization of retroviruses, resulting in the establishment of ERVs, has not been elucidated. The koala retrovirus (KoRV), found in koalas *(Phascolarctos cinereus)*, is a gammaretrovirus which is believed to be currently endogenizing into its host, thus providing us with a rare opportunity to investigate the mechanisms involved in retrovirus endogenization (Stoye, 2006; Tarlinton *et al*., 2006). Genetically and phylogenetically, KoRV is closely related to gibbon ape leukemia virus (GALV) which is an exogenous gammaretrovirus and induces leukemia/lymphoma in gibbons (Delassus *et al*., 1989). In addition, KoRV shares the viral receptor (Pit-1, a phosphate transporter) with GALV when it infects cells (Oliveira *et al*., 2007).

In addition to benefits provided by ERVs, there are also negative consequences of harboring them in the host genome. Indeed, increased levels of KoRV infection in koalas have been associated with several diseases. For instance, koalas suffer from leukemia and lymphoma with a rate of 3–5% in the wild and an even higher rate of up to 60 % in some captive colonies (Canfield *et al*., 1988; Hanger *et al*., 2000). Tarlinton et al. reported that, using quantitative real-time reverse transcriptase (RT)-PCR, KoRV RNA levels in plasma were significantly increased in koalas suffering from leukemia or lymphoma when compared with

healthy koalas (Tarlinton *et al*., 2005). These observations suggest that KoRV is linked to oncogenesis in koalas, making the study of this virus important for understanding its pathogenesis.

Construction and characterization of an infectious clone of KoRV

To date, studies on KoRV infection have been limited due to the lack of a replication-competent molecular clone. Quite recently, we succeeded in constructing an infectious molecular clone of KoRV, termed pKoRV522 (Shojima *et al*., 2013a). It is known that gammaretroviruses bud from the plasma membrane of infected cells. Gag proteins of many retroviruses include short peptide motifs required for virus budding, termed L-domain motifs. To date, three types of L-domain motifs (PT/SAP, PPXY, and YXXL) have been identified. It was reported that the disruption of the PPXY motif in the L-domain of KoRV was involved in the attenuation of KoRV in the process of endogenization into the host (Oliveira *et al*., 2007). Although pKoRV522 has the same mutation in this motif, the virus derived from the pKoRV522 replicated efficiently in human embryonic kidney (HEK) 293T cells, reaching a maximum titer of 10^6 focus-forming units/ml (Shojima *et al*., 2013a). By comparing the Gag sequences of KoRV and GALV, we found an additional intact PPXY motif 18 bp downstream of the PSAP motif in KoRV. By virus budding assays, mutations in the PSAP motif did not affect KoRV budding, whereas mutations in the novel PPXY motif had a significant impact on KoRV budding (Shojima *et al*., 2013a). Therefore, the second PPXY motif is considered to be the major L-domain sequence for the KoRV budding while the first PPXY is dispensable (Shojima *et al*., 2013a).

It has been reported that the PPxY motif interacts with the WW domains of the cellular Nedd4-like E3 ubiquitin ligases (Martin-Serrano *et al*., 2005; Yasuda *et al*., 2002). These host factors are the cellular proteins involved in the multivesicular body (MVB) sorting pathway. The interaction of a viral L-domain with Nedd4-like E3 ubiquitin ligases is essential for the virus budding, and budding of the retroviruses possessing L-domains and MVB vesicle formation might be analogous processes. To further analyze the molecular mechanism of KoRV budding, we examined the involvement of Nedd4-like E3 ubiquitin ligases on the KoRV budding. Consequently, we demonstrated that WWP2 or WWP2-like E3 ubiquitin ligases, possessing the WW domain closely related to WWP2 and Vps4A/B, are involved in KoRV budding (Shimode *et al*., 2013). These data suggest that KoRV Gag recruits the cellular endosomal sorting complex required for transport (ESCRT) machinery through the interaction of the PPPY L-domain with the WW domain(s) of WWP2 and progeny virions are released from cells by utilizing the MVB sorting pathway.

Genomic diversity of KoRVs isolated from Japanese koalas

From 2007 to 2009, we conducted a survey of KoRV infection in koalas in Japanese zoos and succeeded in isolating KoRVs. We identified 4 genotypes whose receptor binding sites are different with each other. By using pseudotype viruses harboring these subgroups, we found that two subtypes (named A and J) infect human cell lines. KoRV-A is similar in nucleotide sequences to the original KoRV clone, termed *pcindy*. The subtype A pseudotype virus shares the receptor with GALV and feline leukemia virus (FeLV) subgroup B (FeLV-B) and utilizes human Pit-1 molecule as a viral entry receptor. The subtype J pseudotype virus utilizes thiamine transport protein 1 (THTR1) to infect human cells as described in the next section. All koalas which are positive for KoRV provirus had KoRV-A in common and many koalas harbor additional subtypes. The long terminal repeat (LTR) of KoRV-J has three tandem repeats in the enhancer region (unpublished data). The promoter activity of LTR of KoRV-J was stronger than that of KoRV-A LTR in HEK293 cells (Shimode *et al*., 2014). The pathological differences in distinct subtypes have not been identified because most Queensland koalas in Japanese zoos are infected with a combination of several subtypes.

Characterization of KoRV-J and prevalence of KoRV-J in koalas in Japanese zoos

Phylogenetic analysis of *env* using the maximum likelihood approach revealed that KoRV isolates and GALV clustered together, but they were distinct from the cluster that consists of FeLVs, murine leukemia viruses (MLVs) and porcine endogenous retroviruses (PERVs) (Fig. 1). KoRVs and GALVs are distantly related to PERVs. Similarities of the Env amino acids among the KoRV-A isolates were shown to be high, and the degree of diversity between KoRV-A and KoRV-J was less than those of FeLV and PERV subgroups.

To further characterize the receptor usage of KoRV-J, we conducted a receptor interference assay using six gammaretroviruses which utilize different receptors, namely, FeLV-A, -B, and -C, RD-114 virus, xenotropic murine leukemia virus (X-MLV) and A-MLV. We found that *lacZ*(KoRV-J) pseudotype virus interfered with FeLV-A on FEA cells (feline fibroblast). The receptor for FeLV-A is known to be thiamine transport protein 1 (THTR1). *lacZ*(KoRV-J) and *lacZ*(FeLV-A) infected *Mus dunni* tail fibroblast (MDTF) cells expressing human THTR1 (hTHTR1), but not naïve MDTF cells. These data indicate that KoRV-J utilizes THTR1 as a receptor (Shojima *et al*., 2013b).

To investigate the prevalence of KoRV subtypes in koalas reared in Japanese zoos, in 2007 to 2009, we collected heparinized blood samples of 40 northern koalas (Queensland, New South Wales and hybrids of Queensland and New South Wales koalas) and 11 southern (Victorian) koalas from 9 zoological parks in Japan, and performed differential PCR analysis using subgroup-specific primer sets. KoRV-A was detected in all northern koalas tested and 4 out of 11 Victorian koalas (Shojima *et al*., 2013b), consistent with previous reports that KoRV had endogenized in koalas in northern Australia. In contrast, KoRV-J was detected in 67.5 % of northern koalas, but not in southern (Victorian) koalas (Shojima *et al*., 2013b). These data indicate that the prevalence of KoRV-J is more limited than KoRV-A, and the invasion of KoRV-J into the koala population may have occurred more recently than KoRV-A.

To determine whether KoRV-J is exogenous or endogenous, we determined the copy numbers of each subgroup in the genomes of different tissues in the individual animals. Copy numbers of each subgroup in tissues of two Queensland koalas (KoRV-A positive, KoRV-J positive) were measured by quantitative real-time PCR. Approximately 3–6 copies of KoRV-A per cell were present in the tissues tested (Shojima *et al*., 2013b). In contrast to the relatively constant copy numbers of KoRV-A among tissues, the copy numbers of KoRV-J were less than 1 copy per cell and varied in tissues in both koalas (Shojima *et al*., 2013b). These data suggest that KoRV-J is not an ERV, at least in these two koalas.

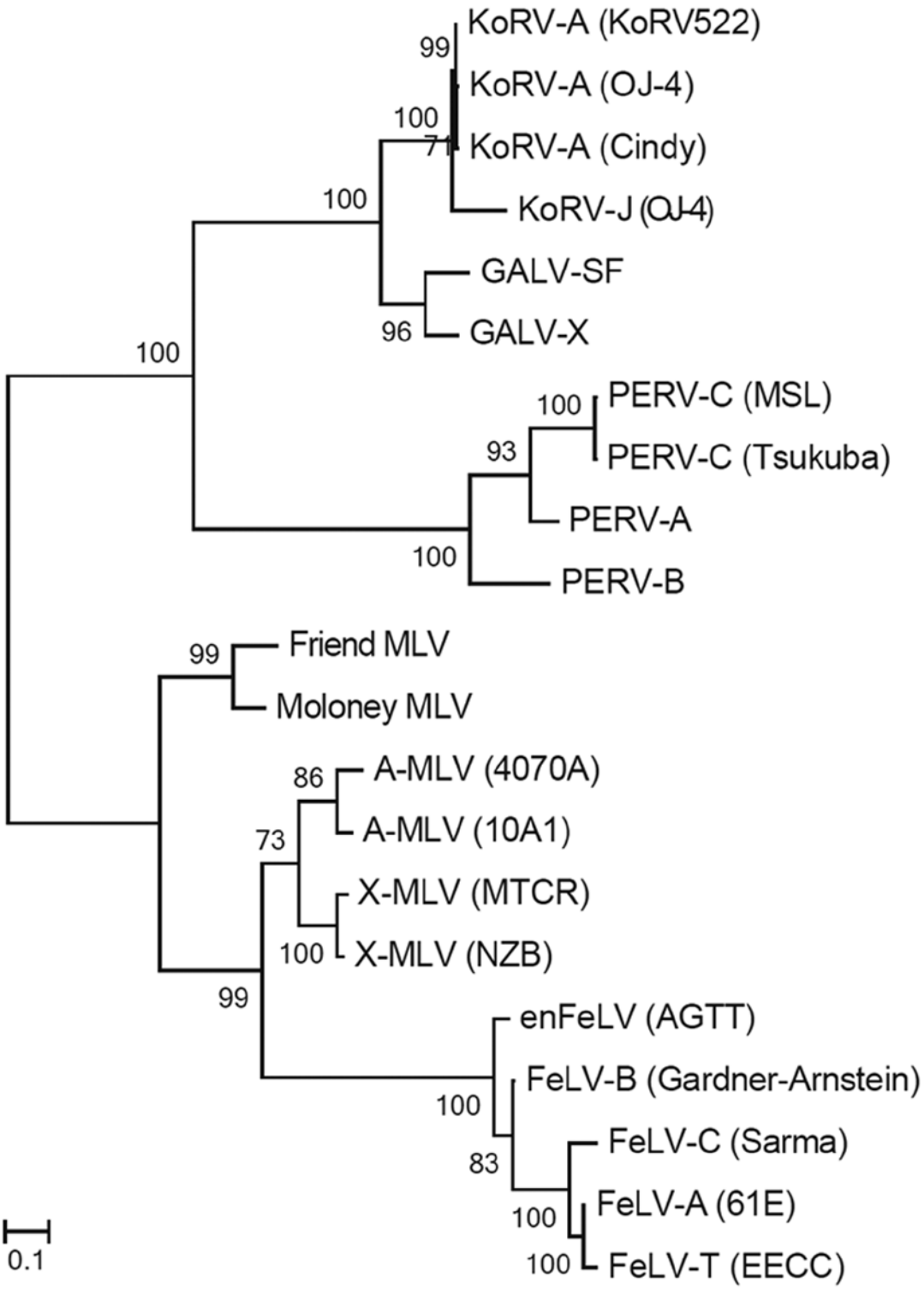

Figure 1. Maximum likelihood tree of the entire amino-acid sequences of *env* genes of KoRV isolates and other gammaretroviruses. Numbers at the nodes indicate percent of rapid bootstrap values (1000 replicates). Amino acid sequences used for the analyses were retrieved from the GenBank database. Abbreviations: A-MLV, amphotropic MLV; X-MLV, xenotropic MLV; enFeLV, endogenous FeLV.

It is plausible that KoRV-J-infected northern koala(s) were introduced into Japanese zoos rather than the virus being derived from other animals within the facilities, especially given that koalas are kept separately from other animals except humans. The origin of KoRV-J is unknown at present. The low amino acid similarity on the surface of Env was not simply caused by nucleotide insertions and/or deletions. Furthermore, it is unlikely that KoRV-J was generated from KoRV-A due to an accumulation of nucleotide mutations. KoRV-J could have been prevalent in an unknown host species in Australia that infected a population of northern koalas quite recently. It is also possible that KoRV-J may be the result of a recombination event between KoRV-A and another KoRV-related gammaretrovirus. Thus far, we have been unable to find any KoRV-J variable region A-like sequences in the NCBI nr/nt database, meaning that further studies are needed to elucidate the origin of the virus. Different receptor usage of KoRV subtypes may explain the wide range of diseases seen in koalas.

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Koala Retrovirus Related Diseases in European Zoo-based Koalas *(Phascolarctos cinereus)*

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Abstract. European zoos have housed koalas *(Phascolarctos cinereus)* for almost 25 years. From the time the first individual arrived on the old continent to the present population of 30 (15.15) animals, medical knowledge has improved significantly. During this time, 57 koala deaths have been recorded. With the discovery of the koala endogenous retrovirus (KoRV), the question remains whether it is involved in the various diseases found in captive population and specifically whether it was involved in the 57 deaths. This question is unfortunately difficult to answer as no real time tests were performed before and during the course of the diseases. A study of the detailed information of these records shows that almost half of them concern very young animals probably mainly because of joeys falling from the pouch and maternal neglect. A few deaths have no recorded information or are clearly not related to any infectious cause. 44% are due to neoplastic and opportunistic or non-opportunistic bacterial infectious process. While KoRV is thought to cause immunosuppression and tumour induction (mainly lymphomas), the link between disease and the virus has not been clearly established.

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The San Diego Zoo loaned the first koalas to European zoos in 1989 (Hamlin Andrus, 2011) to the London zoo. The first breeding pair arrived in 1991 at the Jardim Zoologico de Lisbon, Portugal. Twenty-five years later, seven institutions house 30 animals (15.15) (Hamlin Andrus, 2011).

Over the past 25 years, our knowledge in husbandry and care of this unique species has greatly improved. One of the major recent discoveries was the presence of an active endogenous retrovirus in the genome of a large portion of wild koalas and most (if not all) captive koalas (Canfield *et al.*, 1988; Hanger *et al.*, 2000; Tarlinton *et al.*, 2005). This retrovirus is thought to be responsible for an innate immunosuppression and neoplastic induction, especially, but not only, lymphoma (Tarlinton *et al.*, 2005). This detailed retrospective study of koala deaths that have occurred in the European captive population aims to look for a possible association with the presence of the retrovirus.

In 25 years, 57 deaths have been recorded in the European population (a list of every death with details can be found in Table 1). While every adult death has been examined in detail, some joey deaths, mainly during the first days of life, are missing, depending on the keepers observations.

Among these 57 deaths, 4% (2) have no record. 5% (3) are clearly not related to the retrovirus: one case of bladder stone with death occurring post surgery, one case of head trauma with hydrocephalus internus associated, and one case of ileum and colon torsion.

Deaths of joeys accounted for 47% (27) of all deaths. Most of these cases have no details, but we can assume most of them are related to joey falling from the pouch or maternal neglect (absence of pouch cleaning, joey rejected, etc.). A few cases have bacterial culture recorded and the results are listed among the other bacterial results (Table 2).

Table 1. European captive koala deaths. *institution,* where death recorded; *SB*, studbook number in the International studbook.

institution	SB	sex	birth date	death date	death information
LISBON	116	female	07/06/82	20/07/00	Presumably due to pelvic infection but not possible to determine the origin. Septic metritis. Bladder suppurative inflammation. Arteriosclerosis. Splenic atrophy.
LISBON	117	female	14/07/82	09/11/92	Lymphoid neoplasia—lymphoblastic lymphosarcoma in the abdominal cavity.
DUISBURG	120	male	18/05/83	08/07/04	Unknown cause of death.
LONDON	124		13/06/84	07/11/91	Disseminated lymphoma.
LISBON	148	male	04/06/89	26/12/07	Large Intestine adenocarcinoma—glandular proliferation of the mucosa and submucosa.
DUISBURG	173	male	20/07/92	31/08/05	Moderate alveolar lung emphysema, gastritis, lymphadenitis of mesenterial lymph nodes, volvulus, ulcerative, haemorrhagic and necrotising colitis, focal necrosis of gall bladder, myocardial degeneration.
LISBON	176	female	26/09/92	08/02/93	Malnutrition during her last month on the pouch.
LISBON	184	female	09/06/93	13/12/93	Unknown cause of death (joey).
DUISBURG	185	female	01/07/93	08/10/07	Lungs: focal acute purulent pneumonia *(Enterobacter cloacae, E.coli)*, partly subacute to chronic granulomatous parabronchial lesions with storage of crystalline structures.
DUISBURG	194	male	16/05/94	22/09/04	Unknown cause of death (joey).
LISBON	195	unknown	15/06/94	25/06/94	Rejection by the mother.
LISBON	200	female	14/10/94	15/01/02	Renal carcinoma—tumoral mass (9×5 cm) in the left perirenal area.
DUISBURG	201	male	26/10/94	18/05/06	Alveolary lung emphysema, focal purulent-necrotizing esophagitis, haemorrhagic gastroenteritis, fatty liver, interstitial fibrosis of pancreas.
BEAUVAL	204	male	24/05/95	11/01/07	Polymorphous lymphosarcoma (mesenteric lymph nodes, lungs, liver, bone marrow). Typhlocolitis.
DUISBURG	206	unknown	07/06/95	07/06/95	Unknown cause of death (joey).
PLANCKNDL	212	female	21/10/95	11/04/04	Fatty degeneration of the liver with necrosis, pneumonitis and atrophy of lymphoid tissues.
DUISBURG	224	unknown	01/01/97	18/02/97	Unknown cause of death (joey).
PLANCKNDL	225	male	29/03/97	13/08/01	Leukaemia.
DUISBURG	239	unknown	29/03/98	29/03/98	Unknown cause of death (joey).
LISBON	242	unknown	06/06/98	09/06/98	Foetus found on the floor, replaced. Disappeared next day.
PLANCKNDL	250	male	09/03/99	27/06/07	Chronic proliferative lymphoid inflammatory process. Liver, pancreas, abdominal fluid: *Enterobacter* sp., *Citrobacter* sp.
PLANCKNDL	253	unknown	17/05/99	23/05/99	Unknown cause of death (joey).
LISBON	256	female	27/06/99	28/12/99	Lung: oedema, mild to moderate. Liver: congestion, moderate, with possible individual cell necrosis.
DUISBURG	260	unknown	06/11/99	09/11/99	Unknown cause of death (joey).
PLANCKNDL	262	unknown	18/11/99	15/03/00	Unknown cause of death (joey).
DUISBURG	270	female	03/04/00	06/07/01	Cervical tumours, aspiration pneumonia, infarctions in left heart chamber, liver necrosis centrolobullary.
LISBON	272	female	06/05/00	03/06/03	Serosal tumour—generalized sarcoma (nodules in the stomach, intestines, peritoneum) from fusiform cells originally from peritoneal serosa.
BEAUVAL	269	female	01/06/00	22/03/08	Septicaemia and suspected mesenteric lymphoma.
VIENNA	297	male	24/02/01	26/04/08	Colon leiomyosarcoma, colon stenosis, osteolysis carpus.
MADRID Z	298	male	10/03/01	08/11/09	Multicentric lymphoid leukaemia.
DUISBURG	299	male	17/03/01	17/11/07	Hydrocephalus internus, degenerative changes in the cortex and massive subacute degenerative/necrotising changes of the skeletal musculature most probably caused by trauma.
PLANCKNDL	309	female	02/02/02	20/09/12	Ulcerative membranous necrotic enteritis-cecitis. Lymphoid hyperplasia.
PLANCKNDL	338	female	10/07/02	11/10/03	Diffuse necrotizing hepatitis, strong suspicion of ulcus, glomerulonephrosis, exhaustion of the immune system. *Pseudomonas aeruginosa* (liver, lung, brain, intestinal ulcer).
DUISBURG	343	unknown	13/11/02	13/11/02	Unknown cause of death (joey).
LISBON	344	unknown	27/12/02	03/06/03	Death during hand rearing process.
DUISBURG	349	unknown	26/06/03	28/03/04	*Pneumocystis carinii* in lungs and brain.

Table 1 *(continued)*.

institution	SB	sex	birth date	death date	death information
DUISBURG	353	male	12/08/03	25/10/10	Severe purulent and emphysematous prostatitis, high amount of neutrophil granulocytes. *E.coli haemolytica* (+++) and *Klebsiella pneumoniae* (+).
PLANCKNDL	354	unknown	13/08/03	15/08/03	Unknown cause of death (joey).
PLANCKNDL	358	unknown	24/11/03	03/02/04	Unknown cause of death (joey).
LISBON	362	male	28/05/04	22/04/11	Malignant round cells tumour—right nasal cavity with infiltration of retro-orbital area, right frontal lobe, mandibular lymph node, adrenal gland and prostate.
PLANCKNDL	364	unknown	22/08/04	06/10/04	Unknown cause of death (joey).
DUISBURG	372	unknown	20/06/05	21/06/05	Unknown cause of death (joey).
DUISBURG	375	female	03/10/05	06/10/09	Bladder stone. Died post surgery. Sepsis and circulatory failure due to torsion. *Citrobacter freundii*. Tubuli calcification.
PLANCKNDL	397	male	16/05/06	28/10/08	Toxoplasmosis.
MADRID Z	398	unknown	06/06/06	01/12/06	Consolidated area on the lungs (aspiration pneumonia or septicaemia) and brain oedema (septicaemia). *Klebsiella pneumoniae* from all samples (pouch incl.).
MADRID Z	409	female	12/05/07	04/02/08	Found dead on the floor. *Enterobacter intermedius*.
PLANCKNDL	408	female	12/05/07	29/10/08	Toxoplasmosis.
MADRID Z	417	female	30/08/08	04/03/09	Joey fell several times from pouch. Found dead. Spleen, liver and kidney: *Pseudomonas fluorenscen*; lungs: *Moraxella* sp.
BEAUVAL	418	female	18/11/08	07/12/10	Multicenter lymphoma and leukaemia. Severe lipidosis and haemosiderosis.
MADRID Z	426	unknown	21/04/09	29/08/09	Unknown cause of death (joey).
MADRID Z	434	male	10/10/09	13/09/10	Ileum and colon torsion. Cecum and proximal colon vascularization had congestive mucosa with haemorrhagic areas. Emphysema in the distal area of the lungs. *E. coli* in liver and intestine.
DUISBURG	439	unknown	06/06/10	06/09/10	Unknown cause of death (joey).
DUISBURG	446	unknown	25/10/10	25/10/10	Unknown cause of death (joey).
PLANCKNDL	?	unknown	28/06/11	29/06/11	Unknown cause of death (joey).
PLANCKNDL	?	unknown	22/08/11	29/08/11	Unknown cause of death (joey).
MADRID Z	?	female	22/09/11	02/04/12	Neutrophilic margination in the brain suggests terminal endotoxemia/bacteraemia; isolation of *Klebsiella* from the spleen and *E. coli/Staphylococcus* from kidney.
PLANCKNDL	?	unknown	10/08/12	01/09/12	Unknown cause of death (joey).

Deaths associated with neoplasms accounted for 23% (13) of all deaths. Among those, six cases are lymphoma and one case a leukaemia. These cases present a high suspicion of association with KoRV. The remaining neoplastic processes are leiomyosarcoma, malignant round cell tumour, adenocarcinoma, generalized sarcoma, renal carcinoma, and an unidentified cervical tumour. Whereas these tumours have never been described in association with KoRV, we cannot exclude the relationship. Retroviruses have been described in all mammals including humans being directly associated with different type of carcinoma, sarcoma, and lymphoma. Human immunodeficiency virus (HIV) is indirectly associated with carcinoma and sarcoma of various organs and leiomyosarcoma (Gessain, 2013).

5% (3) of deaths are described as being caused by an opportunistic infection. Two cases of toxoplasmosis with tachyzoites of *Toxoplasma gondii* found on histology and one case of lung and brain infection by *Pneumocystis jirovecii (carinii)*. Toxoplasmosis is opportunistic in many mammal species, but marsupials are known to be specifically sensitive (Bultel *et al.*, 2013). *Pneumocystis jirovecci* is described to be opportunistic in any species (Dugdale *et al.*, 2013). The relationship of these opportunistic diseases with the immunosuppression caused by KoRV can be suspected in these cases.

Finally 16% (9) of deaths were associated with bacterial infections, with pneumonia most predominant. A list of all bacteria cultured can be found in Table 2. Some of these bacteria are often described as opportunistic and the others are generally found as part of the normal intestinal flora (Euzéby, 2013). Again immunosuppression caused by KoRV is suspected.

In conclusion, we can say that 44% (25) of the 57 deaths recorded during 25 years of captive management of koala in Europe could potentially have been related to KoRV. However, as no tests have been performed on the population, and no viral load kinetics on animals before and during the course of disease exist, it is not possible to definitively associate any of these deaths with the virus. It is also difficult to make the distinction between naturally occurring disease and induced disease as the population and number of cases are too small to statistically correlate this with what can be found in other species. Although not definitive, based on the current knowledge of KoRV in koalas there is a high index of suspicion that the seven cases of death due to lymphoma/leukaemia in this retrospective study may have been associated with the virus.

Table 2. List of bacteria cultured at necropsy.

Bacteria name	Gram staining	Family	Opportunistic status
Pseudomonas sp. incl. *aeruginosa* & *fluorenscen*	Gram –		opportunistic
Enterobacter sp. incl. *intermedius* & *cloacae*	Gram –	Enterobacteriaceae	opportunistic
Citrobacter sp. incl. *freundii*	Gram –	Enterobacteriaceae	
Escherichia coli incl. *haemolytica*	Gram –	Enterobacteriaceae	
Klebsiella pneumoniae	Gram –	Enterobacteriaceae	
Staphylococcus sp.	Gram +		opportunistic
Moraxella sp.	Gram –		opportunistic

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Retrovirus-related Disease in Zoo-based Koalas *(Phascolarctos cinereus)* in North America

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Abstract. Koala retrovirus (KoRV)-related disease is a major suspected cause of death in koalas *(Phascolarctos cinereus)* in zoos in North America. There are currently eleven zoos exhibiting koalas in North America. A mortality survey of these institutions indicated that mortalities directly related to KoRV (e.g., lymphoma, leukemia, anemia, bone marrow hypoplasia, osteochondromatosis) and mortalities suspected to be KoRV-related (e.g., immunosuppression, unusual opportunistic infections [e.g., Coccidioidomycosis], potentially other neoplasia) account for 41% of deaths. Testing of the living North American koala population for a recently reported, exogenous koala retrovirus variant (KoRV-B) identified four KoRV-B-positive individuals in a population of 54 koalas (7.4%).

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Koalas (*Phascolarctos cinereus*) have been exhibited in North America since 1925 with the North American regional studbook tracking koalas since 1971. Koala retrovirus (KoRV) has been suspected to be a major cause of mortality in some zoo-based koala populations in south-eastern Queensland, Australia, where it has been reported anecdotally to cause up to 80% of mortalities (Hanger *et al*., 2000). The incidence of mortality related to KoRV in US-based koalas has not been previously reported. In 2011, a novel variant of KoRV (KoRV-B) was reported following a number of malignant cancers and deaths related to KoRV in koalas at the Los Angeles Zoo (Xu *et al.*, 2011; Xu *et al*., 2013). KoRV-B appears to be exclusively exogenous, unlike the originally sequenced endogenous KoRV-A which is both exogenous and endogenous (Xu *et al*., 2013). The prevalence of KoRV-B has not been previously reported in the US and is currently unknown in Australia.

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Methods and materials

A KoRV mortality survey was emailed in February 2013 to veterinarians at nine of the eleven institutions in North America currently exhibiting northern koalas. Two zoos were not emailed surveys due to only recent acquisitions of northern koalas with no deaths. All nine emailed-institutions responded (Table 1).

Fresh-EDTA treated blood samples were collected from koalas currently living in ten zoos in the US. One zoo (Miami Metro Zoo) was excluded due to the geriatric, non-reproductive age of their two koalas. Samples were sent at room temperature overnight and tested for the presence of KoRV-B by PCR using plasma and peripheral blood mononuclear cell DNA (Table 2).

Table 1. Deaths related directly or suspected to be related to KoRV in northern koalas *(Phascolarctos cinereus)* at US zoos.

institution	number of deaths[a]	direct KoRV[b]	suspect KoRV[c]	total %
San Diego Zoo[f]	124	36	13	40
Los Angeles Zoo	7	4	1	71
San Francisco Zoo	14	2	2	29
Riverbanks Zoo and Garden	5	2	0	40
Cleveland Metroparks Zoo[d]	8	2	4	75
Columbus Zoo and Aquarium[d]	7	1	0	14
Miami Metro Zoo[d]	1	1	0	100
Lowry Park Zoo[d,e]	3	0	1	33
Albuquerque Zoo[e]	0	n/a	n/a	n/a
Dallas Zoo[d]	0	n/a	n/a	n/a
Palm Beach Zoo[e]	0	n/a	n/a	n/a
total	169	48	21	41

a Deaths with necropsy and histopathology results.
b Deaths directly associated with KoRV (e.g., lymphoma, leukemia, anemia, bone marrow hypoplasia, osteochondromatosis).
c Deaths suspected to be associated with KoRV (immunosuppression, unusual opportunistic infections, e.g., coccidioidomycosis, other neoplasia).
d San Diego Zoo origin koalas.
e Los Angeles Zoo origin koalas.
f Includes koalas on loan to other non-named zoological institutions.

Table 2. KoRV-B testing of living koalas *(Phascolarctos cinereus)* at US zoos (February 2013).

institution	number of koalas tested	number of koalas not tested	KoRV-B	total %
San Diego Zoo	14	5 (incl. 3 joeys)	0	0
San Diego Zoo Quarantine	3	0	1	33
Los Angeles Zoo	6	1 (joey)	0	0
San Francisco Zoo	4	0	0	0
Riverbanks Zoo and Garden	2	2 (incl. a joey)	0	0
Cleveland Metroparks Zoo[a]	1	5	0	0
Columbus Zoo and Aquarium[a]	2	0	0	0
Miami Metro Zoo[a]	0	2	—	—
Lowry Park Zoo[a]	1	0	0	0
Albuquerque Zoo[b]	2	0	2	100
Dallas Zoo[a]	2	0	0	0
Palm Beach Zoo[b]	2	0	1	50
total	39	15	4	7.4

a San Diego Zoo koalas
b Los Angeles Zoo koalas

Discussion

As is the situation in some zoo-based koala populations in Australia, KoRV is likely a significant cause of mortality in koalas in the US with it associated with up to 41% of all mortalities. These results highlight the clinical importance of this virus and that the sustainability of the population could be greatly increased if measures to reduce the expression of KoRV-related disease could be discovered.

The significance of the presence of KoRV-B in some zoos in the US is unknown at this time. Further work is required to determine the pathogenicity of KoRV-B and whether it is of any more concern than KoRV-A. Extensive testing in Australia coupled with mortality reviews may answer these questions. The findings could determine the relative importance of a KoRV-B-negative koala population and could negatively impact the sustainability of the US koala population if there is a need to keep KoRV-B-positive and KoRV-B-negative koalas separated.

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How does Koala Retrovirus (KoRV) Induce Disease at the Genomic Level?

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Abstract. This manuscript summarizes the break-out session held on how does koala retrovirus (KoRV) induce disease at the genomic level at the *Koala Conservation Workshop: The koala and its retroviruses: implications for sustainability and survival* held at San Diego Zoo, April 17–18, 2013. The goals of this break-out session were to review current knowledge in this area and identify studies required to fill important gaps. KoRV is a gammaretrovirus with close similarity to MLV and FeLV, well-characterized pathogens of the laboratory mouse and the domestic cat. The parallel wth FeLV is particularly striking as cats harbor related endogenous retroviruses that share receptor specificity with endogenous KoRV. Also, transmission and pathogenesis of FeLV in its natural host is well understood and the virus is routinely controlled by measures that include vaccines. Alternative models for the roles of endogenous and exogenous KoRV in disease were discussed and prospective studies required to test these hypotheses were outlined.

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Introduction

What do we know? Most koala populations contain integrated KoRV-A. It appears that a subpopulation of koalas e.g. on Kangaroo Island (KI) may be free of KoRV, although available data based on PCR and hybridization analysis with KoRV-specific probes cannot be regarded as a definitive negative. The prevalence of disease appears to correlate with the copy number of KoRV, high in Queensland and low in other areas such as KI. Southern blot analysis of integrated KoRV from "high copy number" Queensland koalas reveals a similar pattern across tissues suggesting that most or all KoRV copies are germ-line rather than somatically acquired.

The length of time KoRV has been in the koala population is unclear but the recovery of integrated KoRV from koala skins in museum collections suggests that the infection may be older than previously supposed. However, the remarkably high copy number in some koala populations suggests that expansion of endogenous KoRV sequences may be more recent.

Analysis of lymphomas from captive koalas in US zoos has revealed the presence of variant KoRV with altered host range (KoRV-B, C) due to mutational changes in the viral *env* gene. They also show duplications of the core enhancer sequences in the viral LTR. Similar changes have been observed previously in murine and feline gammaretroviruses and are associated with increased replication in lymphoid tissues and leukemogenicity. These features suggest that KoRV induces lymphoma by an insertional mutagenesis mechanism similar to other gammaretroviruses. There is a further remarkable parallel between KoRV and feline leukemia virus (FeLV). Endogenous FeLV-related sequences, which are ancient (c. 6 million years) and invariably replication defective, encode an envelope protein that binds Pit-1, like KoRV-A. The prevalent infectious form of FeLV, FeLV-A, utilizes THTR1, like KoRV-B. FeLV-A recombines with endogenous FeLV to generate FeLV-B, and such recombinant viruses are more common in leukemic cats. However, the KoRV variants appear to arise by limited mutations from KoRV-A, presenting a challenge to the development of simple molecular typing and detection methods such as those used to analyse *de novo* integrated MLV and FeLV on a complex background of related endogenous viruses.

Gaps in knowledge

1 If it could be shown that KoRV is capable of infecting somatic cells and induces lymphoma by insertional mutagenesis, this would firmly establish its role in disease and argue in favour of measures to limit transmission and dissemination. However, although the parallels with other gammaretroviruses are persuasive, direct evidence is lacking. Lack of koala genome sequence data is another significant constraint.

2 Based on existing data, three main scenarios are possible:

- 2.1 Endogenous KoRV (KoRV-A) is unable to re-enter somatic cells due to defectiveness or interference barriers, with KoRV-related disease arising due to superinfection with horizontally transmitted forms such as KoRV-B (the FeLV model)
- 2.2 Endogenous KoRV is capable of replication, leading to evolution of more pathogenic forms within an individual animal (the Akv model).
- 2.3 An intermediate situation where KoRV-B and other variants arise occasionally by mutation and are then transmitted to koalas in contact, either horizontally or via milk to offspring.

It will be important to distinguish between these possibilities as they have significantly different implications for disease prevention and control.

3 Another deficiency is the lack of information on immune responses to KoRV. This is important to establish whether control by vaccination will be feasible. Specifically :

- 3.1 Do apparently KoRV-negative koalas make immune responses due to exposure to infected animals?
- 3.2 Does expression of germ-line KoRV-A lead to immune tolerance and susceptibility to *de novo* infection with more pathogenic strains (e.g., KoRV-B)?

4 Innate/intrinsic immunity to KoRV has not yet been examined.

Major questions to be addressed

Analysis of *de novo* integrations and somatic mutations in lymphomas of KoRV-infected koalas will require the collection of uninvolved tissue as well as tumor at post-mortem. Disease arising in zoos offers the best prospect of obtaining fresh port-mortem tissues and should be prioritized. There are problems with adopting methods used in the mouse, as high copy numbers of endogenous KoRV may obscure *de novo* integrations, and the precise genomic location is unlikely to be clear in the absence of koala genome sequence. A more accessible though indirect method of testing the insertional mutagenesis hypothesis would be to look by Southern blot analysis for rearrangements in the homologues of known lymphoma target genes that are common to other gammaretroviruses across species (e.g., *Myc, Gfi1, Pim1, Myb*). Probes derived from conserved coding sequences of the genes should be first tested for their ability to detect unique sequences in the koala genome by blot analysis. PCR amplification of specific exons could also be used to generate higher specificity. If rearrangements are found, further restriction enzyme digests and/or direct PCR amplification and sequencing could then be used to demonstrate the presence and location of newly integrated KoRV.

1 Tumour typing is limited due to relative lack of surface phenotype markers. Demonstration that tumours are clonal expansions of T or B-cells could be achieved using conserved probes from TCR or IgH loci. Again, cloning of conserved exons from koala orthologues should be straightforward.

2 There is a need to characterize KoRV isolates further and establish whether KoRV-B/J or other variants are essential for disease development. This will require virus isolation from healthy and diseased animals and analysis of tropism. It is known that KoRV-A and B can infect and replicate in permissive human cells (e.g., HEK293) but the possibility that other *env* variants may be unable to replicate in these cells should also be considered. Development of primary fibroblast cultures from koalas would be advantageous if this can be achieved (e.g., from non-viable joeys).

3 Sequence analysis of KoRV-B/J variants from multiple sources may give clues to the frequency of occurrence and transmission e.g., geographical localization of unique signature sequences of variants would indicate local transmission rather than de novo generation.

4 Analysis of multiple tissues from postmortem samples of koalas with lymphoma or other diseases would indicate whether variant KoRV are present in germ-line or somatically acquired.

5 A neutralization test for KoRV would be helpful for analysis of specific immune responses. A suitable assay could be generated using pseudotype viruses (lacZ/GFP). Western blot analyses would complement these studies, but will require anti-koala Ig.

6 The unusual germ-line amplification of KoRV-A in Queensland koalas is of potential significance. The possibility that koalas are deficient in restriction factors that confer innate or intrinsic immunity to retroviral spread (e.g., the APOBEC family) could be investigated by cloning and functional analysis of koala orthologues of this and other relevant gene families. It would be important to determine whether e.g. southern koala populations are more intrinsically resistant to KoRV despite their relative lack of reproductive fitness.

Resources required:

- Well annotated KoRV isolates from healthy and diseased koalas.
- Control and tumour tissues from diseased animals.
- Specific probes for likely target genes for insertional mutagenesis and koala TCR/Ig.
- Koala fibroblast cultures (if feasible) to examine KoRV growth properties in natural host cells.
- Sera from koalas apparently lacking KoRV and KoRV positive controls.
- DNA from divergent koala populations for restriction factor cloning and analysis.

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Koala Retrovirus (KoRV) and its Variants

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Abstract. The recent, independent identification by several research groups of koala retrovirus (KoRV) variants was the focus of one of the break-out sessions at the *Koala Conservation Workshop: The koala and its retroviruses: implications for sustainability and survival* held at San Diego Zoo, April 17–18, 2013. The goals of this session were to discuss the differences and similarities between variants identified, to determine approaches to their nomenclature, the prevalence of these variants in wild and captive koalas, the relative pathogenicity of the variants, and the significance of the variants in managing koala populations.

Young, Paul R. 2014. Koala retrovirus (KoRV) and its variants. In *The Koala and its Retroviruses: Implications for Sustainability and Survival*, ed. Geoffrey W. Pye, Rebecca N. Johnson and Alex D. Greenwood. *Technical Reports of the Australian Museum, Online* 24: 59–60.

The nucleotide sequence of a koala retrovirus thought to be associated with lymphoma was first reported by Hanger in 2000 (Hanger *et al*., 2000) and named KoRV. More recently, separate research groups in Australia, Japan, and the United States have independently identified a number of KoRV variants (Miyazawa *et al*., 2011; Xu *et al*., 2013; Shojima *et al*., 2013; Shimode *et al*., 2014; own unpublished observations). At the time of this meeting in April, 2013 the only sequence analysis that had been publicly presented on KoRV variants was by the Miyazawa group at the 21st International Workshop on Retroviral Pathogenesis in Italy, September 2009. They showed that a variant, isolated from a captive koala at the Kobe Municipal Oji Zoo (KMOZ) was characterized by a significant sequence modification in the *env* gene, within the receptor binding domain (RBD), specifically in the variable region A (VRA) motif that is known to be involved in defining host cell receptor specificity. They referred to this variant as KoRV-B with the original Hanger strain designated KoRV-A and published the isolation of these viruses in the following year (Miyazawa *et al*., 2011). Recognition by other groups of variants with differing sequence stretches in this same region of the RBD VRA has led to the adoption of the Miyazawa labeling convention. Discussions during this break-out session at the 2013 San Diego meeting didn't reach consensus on KoRV nomenclature nor on a number of other issues raised, primarily because none of the sequences had at that stage been published and so direct comparative analyses could not be made. However, subsequent publications have helped to clarify the situation and the discussion below is intended to summarize the current state-of-play.

KoRV variant nomenclature

The natural extension of the above naming convention has resulted in the publication so far of five KoRV variants with the original sequenced virus being designated KoRV-A and the remaining four being named KoRV-B, KoRV-C, KoRV-D, and KoRV-J (summarized in Denner & Young, 2013). The original Miyazawa KoRV-B had to be re-named KoRV-J as an isolate from the Los Angeles Zoo (LAZ) was given the KoRV-B designation in the first published sequence analysis of a KoRV variant (Xu *et al*., 2013). Ironically, subsequent sequence comparisons indicate that the LAZ KoRV-B VRA sequence is strikingly similar to KoRV-J placing these two viruses in the same phylogenetic grouping (Shimode *et al*., 2014). Furthermore, both of these viruses were shown to utilize the same receptor, the thiamin transport protein 1 (THTR1) for cell entry, a different receptor to that used by KoRV-A, the sodium-dependent phosphate transporter, Pit1 (Shojima *et al*., 2013; Xu *et al*., 2013). Full genome sequencing of these isolates has identified additional sequence variation from the prototype KoRV-A with both KoRV-B and KoRV-J showing additional, but distinct tandem repeats in the U3 region of the LTR (Shimode *et al*., 2014). Interestingly, the KoRV-J LTR was shown to display a significantly higher promoter activity than the KoRV-A LTR in selected cell populations hinting at a possible role in up-regulating the expression of host cell genes adjacent to proviral insertions (Shimode *et al*., 2014). Given the striking similarities between KoRV-B and KoRV-J it would be appropriate for both to be referred to as KoRV-B but each with a strain designation to separately identify them

(e.g., KoRV-B strains LAZ and KMOZ). Of particular note is the fact that all variants so far identified have only been found in animals that are also carrying KoRV-A. An obvious conclusion is that the deletions/insertions found in the same *env* location (RBD VRA) for all the variants are the products of a recombination hot spot.

Another convention that has been adopted was discussed at the meeting, that of referring to these isolates as "subtypes". As we probe further into both the koala genome and the KoRV variants that arise in individual animals, we are likely to detect many more of these variants. However as each new isolate is given a subtype listing we may generate unintentional nomenclature conflicts. Sequencing based approaches to virus taxonomy usually define genotypes as the higher order of classification with individual subtypes falling within these. The phylogenetic analysis provided by Shimode *et al*. (2014) of the currently available published sequences suggests three genotypes comprising multiple subtypes; KoRV-A, KoRV-B (containing both the LAZ and KMOZ viruses) and KoRV-C (clustering both KoRV-C and KoRV-D). The close genetic relationship between the KoRV-C and KoRV-D sequences suggests that they could be included in the same KoRV genotype but as separate subtypes. The field needs to have the discussion on selecting the appropriate consensus criteria for classification soon, so that the nomenclature does not become too messy.

An additional issue that needs to be considered, one that is unique to retroviruses, is the notion of virus isolation as a necessary criterion for defining genotypes/subtypes. This is not a scenario that needs to be addressed for most viruses where modern PCR based genotyping does not require virus isolation. Indeed, modern pathology laboratory diagnostics often rely almost solely on molecular detection, resulting in few viruses that have been reported in the literature ever having been isolated or cultured in a laboratory. However with retroviruses it is likely that many variant sequences will be identified following PCR of endogenized elements that may have arisen through recombination *in situ*. These may, or may not give rise to viable replicating viruses. As it happens, all of the reported KoRV sequences noted above have been derived from cultured viruses and so represent true genotypes/subtypes. Perhaps any new variant sequences derived only by PCR of extracted nucleic acid from koala tissue and/or blood should simply be referred to as variants, pending association with a replicating virus.

KoRV variant prevalence in wild and captive populations

The vast majority of wild type sequences that have been generated and deposited in online databases are KoRV-A. All of the published literature reporting variant sequences to date has been generated from viruses isolated from koalas in captivity. Consequently, there is little information available on the spread of these and other variants in the wild Australian koala population. However it is certainly interesting that the KoRV-B and KoRV-J sequences are so similar, given their isolation from geographically separated koalas, suggesting a common infectious ancestry rather than *de novo* generation in their respective individual hosts. Ongoing studies in our laboratory are examining the presence of such variants in wild populations. Intriguingly, a variant we identified and sequenced in 2007 from a koala sampled on Kangaroo Island, off the south coast of Australia, is remarkably similar to KoRV-C, isolated from a koala at the Kobe Zoo and sourced from Queensland. This suggests a broader distribution of these variants in the wild than originally suspected, unless koalas from widely geographically distinct origins were brought together in a captive setting allowing horizontal transmission. Further testing of samples collected in the field will be required to answer this very important question.

KoRV variant pathogenicity

A key question that lifts the discussion of these variants from an interesting taxonomic and evolutionary debate to one of critical importance to koala management is whether particular variants/subtypes are linked to more severe disease outcome. The study by Xu *et al.* (2013) directly examined two captive koala populations in the USA, one highly inbred colony where disease was uncommon and one where new animals were regularly introduced from Australia but where malignant neoplasias were noted. KoRV-B was isolated from animals from the latter but not former colonies. Furthermore, in this small data set, half of the animals from which KoRV-B was isolated (3/6) developed malignant lymphoma. The authors concluded that KoRV-B is associated with lymphoma development and that it may be more pathogenic. Given the nature of the likely generation of these particular variants, it is also possible that the isolation of KoRV-B may simply be a surrogate marker for increased recombination activity, which in turn could drive the increased pathogenic outcome.

Regardless of the answer to this mechanistic question, it is imperative that further studies are performed to validate this proposed pathogenic role for KoRV-B. Any correlation between the presence of a particular KoRV subtype and malignant disease in koalas will have clear implications for breeding programs that maintain stable koala populations in captivity. In wild populations, determining KoRV subtype prevalence and geographic spread should provide valuable insight into the spread of disease and perhaps offer clues to intervention strategies.

Acknowledgments. The author acknowledges the valuable input provided by other meeting delegates into the deliberations of this break-out session, but takes full responsibility for the views presented in this report.

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Establishing Priorities for Research on the Epidemiology of Koala Retrovirus (KoRV) in Koalas *(Phascolarctos cinereus)*

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Abstract. This manuscript summarizes the break-out session held on the epidemiology of disease expression of koala retrovirus (KoRV) in koalas *(Phascolarctos cinereus)* at the *Koala Conservation Workshop: The koala and its retroviruses: implications for sustainability and survival* held at San Diego Zoo, April 17–18, 2013. The goals of this break-out session were to develop and prioritize specific research goals related to KoRV epidemiology, to identify actions, and to determine the responsible parties and timelines. Identified areas for epidemiologic research include studies in both wild and captive populations. For wild populations, baseline estimates of incidence and prevalence that account for potential biases in surveillance are needed. Landscape-level studies that determine whether KoRV contributes to the decline or stability of wild populations are also a priority. Captive populations with high-quality health data and management records can provide opportunities to identify factors associated with disease expression. These populations may also be pivotal in understanding the clinical importance of different KoRV subtypes.

Witte, Carmel L. 2014. Establishing priorities for research on the epidemiology of koala retrovirus (KoRV) in koalas *(Phascolarctos cinereus)*. In *The Koala and its Retroviruses: Implications for Sustainability and Survival*, ed. Geoffrey W. Pye, Rebecca N. Johnson and Alex D. Greenwood. *Technical Reports of the Australian Museum, Online* 24: 61–63.

Relevant to any epidemiologic study are the two important questions: What do we already know and what are the current gaps in knowledge?

What do we know? There are koala populations with low KoRV prevalence and with no disease expression. The Kangaroo Island population is a good example of lower KoRV prevalence with little disease expression (Simmons *et al.*, 2012). There are also populations with high KoRV prevalence with little disease expression (e.g., St. Bees Island) (Tarlinton *et al.*, 2006; Bill Ellis, pers. comm. 2014). There appears to be a difference in prevalence and disease expression between populations of northern koalas versus southern koalas (Simmons *et al.*, 2012). There also appears to be similar prevalence of KoRV with widely differing prevalence of disease expression between southeastern and central Queensland koala populations (Simmons *et al.*, 2013; Amber Gillett and Sean FitzGibbon pers. comm. 2013). One challenge in Australia is that there have only been a few studies where sampling and testing was limited and opportunistic.

Gaps in knowledge. Currently the important gaps in knowledge are: Does KoRV causes disease in koalas and if so, is it associated with declines? What diseases (e.g., neoplasia) are caused by KoRV in koalas? Are there environmental, social, or other triggers for disease expression? What role do the exogenous and endogenous variants of KoRV play in causing disease? Does KoRV viral load increase with age?

Major epidemiologic questions for wild and captive populations:

Wild populations

1 *What is the baseline prevalence of KoRV and KoRV-associated disease in wild populations? What are the demographic and geographic characteristics of affected populations?*

What data do we need?

1.1 Baseline data: prevalence of KoRV and prevalence of associated disease across widespread geographic areas or across a few intensely-studied populations. Note that intensely studied populations may provide better information on KoRV-associated disease.

1.2 Data on demographics for individuals within the population.

Important considerations

1.3 Surveillance bias: Biases in prevalence estimates can result from surveillance sources that are a non-randomized subsets of the koala population. For example, it is unknown whether differences in prevalence observed across koala populations reflect true differences in prevalence of wild populations or differences in workups of koalas in hospitals, which are often used as disease surveillance sources due to the ease of data attainment (Amber Gillett, pers. comm.). Sources of information should be recorded, appropriate controls identified, and analyses and conclusions should take these potential biases into consideration.

1.4 Standardization of mortality data: veterinary pathologists should be involved to help establish consistency in post-mortem disease surveillance methodology (e.g., collecting the same sets of tissues) and diagnoses.

1.5 Data are needed on disease negative and, if possible, KoRV negative animals.

2 *What is the incidence of KoRV infection and disease in wild populations and is it changing over time?*

What data do we need?

2.1 Capture-recapture studies to measure KoRV and disease status in populations where some animals remain infection-free (e.g. Kangaroo Island).

2.1.1 To estimate incidence of KoRV infection, the study population should be groups with some KoRV-free koalas. The KoRV-free koalas should be followed forward through time to determine the rate of new infections. Baseline data on disease prevalence in the source population should be documented at the time of the study.

2.1.2 To estimate incidence of KoRV-related disease, the study population should be KoRV-positive; individuals with confirmed KoRV infection should be followed forward through time to determine the rate of disease outcomes. KoRV-negative animals should also be followed for the same disease outcomes to estimate rate differences by infection status. Consideration should be given to differences in disease rates across different KoRV subtypes.

2.1.3 These studies could ultimately help estimate the rate of spread in a population and contribute to eventual development of infectious disease models.

2.2 Multi-year prospective disease monitoring could help to determine if disease incidence is increasing.

2.3 Data to collect would include blood samples for KoRV status and virus subtyping, age, sex, source population, and reason for sampling (e.g., specific research project, animal injured and brought to hospital, etc.). Additional health and demographic data can be collected. Location of sampling and GPS coordinates if applicable would also be ideal information to obtain.

Important considerations:

2.4 If the rate of spread is expected to be low, then alternative study designs should be considered in consultation with epidemiologists.

2.5 Longitudinal studies currently in progress, where blood samples are being collected and stored, may help answer these questions.

3 *Is KoRV infection and associated disease a factor contributing to koala declines or is it a factor in maintaining stable population?*

What data do we need?

3.1 Landscape-level data on site-specific population declines

3.2 Landscape-level data on KoRV status and disease prevalence at the same locations

3.3 Other landscape-level factors that may contribute to declines, i.e., potential confounders such as habitat destruction, dog density estimates, roads, etc.

Important considerations:

3.4 A large epidemiology study of this magnitude will be challenging.

3.5 Partnering with biologists already studying wild populations is important for more expedited research.

Captive populations

1 *Is KoRV-B more of a risk to captive populations than KoRV-A? Do we need to be more concerned about KoRV-B (Xu et al., 2013)?*

What data do we need?

1.1 Proportional mortality study of death rates among koalas with varying KoRV subtypes.

1.1.1 Needs to be done at an Australian facility where both variants have been observed.

1.1.2 Complete post-mortem disease surveillance with diagnoses for all animals at risk (not just animals where lesions are present).

1.1.3 Need samples for determining KoRV status. Test for presence/absence of all known strains.

1.1.4 A prospective survey would be ideal. The KoRV status of koalas would be determined (negative, KoRV-A only, KoRV-B only, both KoRV-A & B, other variants) and then koalas would be followed prospectively to determine incidence of KoRV-related disease in the different groups.

1.1.5 If banked data are available, a retrospective study could be used to expedite research.

2 *Are there demographic or management factors that contribute to individual susceptibility related to KoRV-related disease and how does viral load modify disease susceptibility?*

What data do we need?

2.1 Need to determine which factors are most important to focus on.

2.1.1 Ideally, they would be characteristics that could be modified through management (e.g., harem size) or monitored (e.g., age group).

What risk factors are we interested in?

2.2 Age class (e.g., does disease only affect post-reproductive, geriatric animals and is there a relationship between age and viral load?).

2.3 Harem size.

2.4 Genetic relatedness.

2.5 Importation of new animals (e.g., introduction of variants of KoRV).

2.6 Which koalas are housed together with changes captured over time.

2.7 Medical history.

What data do we need?

2.8 Electronic management, medical, and necropsy records.

2.9 KoRV status, subtype, and measures of viral load.

3 *Is the pattern of cyclical expression of KoRV-related disease observed at San Diego Zoo real? Are other factors (e.g., aging, importation) related to the observed pattern?*

What data do we need?

3.1 Electronic management, medical, and necropsy records.

Ideas for working together to tackle these research questions:

- Dr Amber Gillett, Australia Zoo Wildlife Hospital will look into sample banking in Australian facilities.
- Carmel Witte can help design and consult on epidemiology studies.
- San Diego Zoo has banked samples and archived data that may address some of the basic epidemiology questions. Medical data and management data are currently not in electronic form and so person-time is needed to more thoroughly investigate.

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Prevention and Treatment of Koala Retrovirus (KoRV) Infection: Lessons from Studies of AIDS Viruses in Nonhuman Primate Models

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Abstract. The presence of multiple retroviruses in koalas (*Phascolarctos cinereus*), including viruses with exogenous infectious forms that may be associated with malignant disease manifestations, poses challenges for both management of captive populations and species preservation in the wild. The development of antiretroviral medications (ARV) for the treatment of human immunodeficiency virus (HIV) infection is one of the triumphs of modern medicine, and many of these drugs have relatively broad antiretroviral activity, suggesting they might be active against koala retroviruses (KoRVs). However, accumulating experience with the use of these medications in non-human primate (NHP) models of HIV infection and acquired immune deficiency syndrome (AIDS) points out several caveats and provides guidance in attempting to use anti-HIV drugs in the treatment of retroviral infection in nonhuman species. This manuscript reviews that experience from the perspective of potential use of ARVs for prevention and treatment of KoRV infection.

Lifson, Jeffrey D. 2014. Prevention and treatment of koala retrovirus (KoRV) infection: lessons from studies of AIDS viruses in nonhuman primate models. In *The Koala and its Retroviruses: Implications for Sustainability and Survival*, ed. Geoffrey W. Pye, Rebecca N. Johnson and Alex D. Greenwood. *Technical Reports of the Australian Museum, Online* 24: 65–69.

The koala (*Phascolarctos cinereus*) represents a fascinating instance of retrovirus/host species interactions, with geographically high prevalence of an endogenizing retrovirus, provisionally designated koala retrovirus-A (KoRV-A), that is also found in exogenous, pathogenic forms, along with a more recently described distinct exogenous related virus, provisionally designated KoRV-B, that utilizes a different cellular receptor and is associated with malignant hematologic manifestations (Ávila-Arcos et al., 2013; Canfield et al., 1988; Hanger et al., 2000; Oliveira et al., 2007; Shojima et al., 2013; Simmons et al., 2012; Stoye, 2006; Tarlinton et al., 2005, 2006, 2008). These viruses represent a management problem for captive populations, and a challenge for species preservation in the wild. The development of antiretroviral drugs for the treatment of HIV infection has dramatically improved both survival and quality of life for HIV infected individuals, and the relatively broad antiretroviral activity of many of these drugs suggest they may also be active against retroviruses affecting non-human species, such as KoRVs (Oliveira et al., 2007). However accumulating experience with the use of anti-HIV drugs in NHP models highlights important considerations and potential limitations to such use that may help inform efforts to use anti-HIV drugs for the treatment of KoRV infection in koalas (Del Prete & Lifson, 2013). Factors to consider include potency against the target virus (compared to HIV), drug delivery, pharmacokinetics, toxicity and sustainability of treatment. Perhaps the most important consideration is the relationship between the mechanism(s) of action and targets of the drugs considered in relation to the underlying pathogenesis of the disease process of concern.

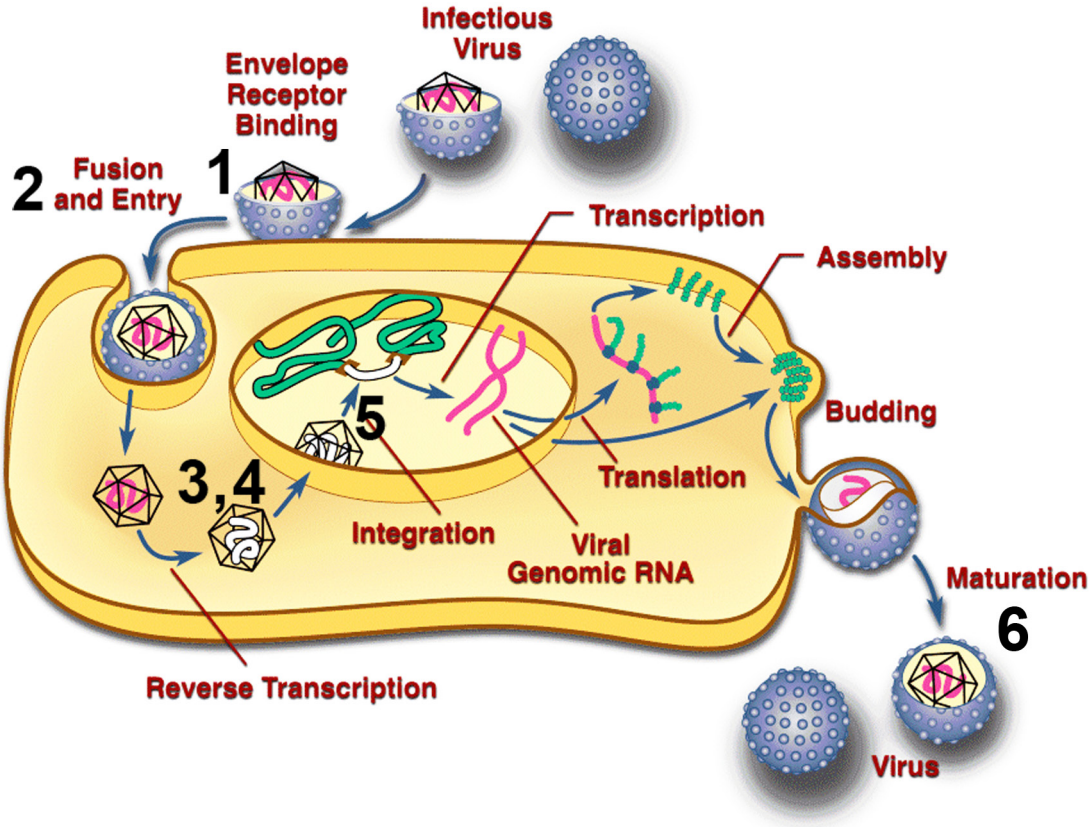

Figure 1. Steps in the retroviral replication cycle present opportunities for therapeutic intervention. Modified from: http://home.ncifcrf.gov/hivdrp/RCAS/images/replication.html

Retroviral replication cycle and drug targets

Retroviruses utilize a host cell dependent, multistep replication cycle for their reproduction that involves extensive interactions with host cell systems (Bieniasz, 2012). Multiple steps in this replication cycle, illustrated in Figure 1, provide potential opportunities for therapeutic intervention, and over the past circa 25 years, a substantial research enterprise has sought to better understand and exploit the therapeutic opportunities presented by these steps.

The retroviral replication cycle is reviewed in detail elsewhere (Bieniasz, 2012). Key steps however are illustrated in Figure 1. (Steps in this replication cycle that are the targets of approved anti-HIV drugs are indicated by numbers in Figure 1): Steps include: binding of mature, cell free virions to receptors and co-receptors on the surface of a susceptible target cell (1), leading to conformational changes that enable facilitated fusion of the membranes of the virion and the host cell (2), entry of the virion contents into the cytoplasm, reverse transcription of the viral RNA genome into DNA (3,4, reflecting two different drug classes targeting the HIV reverse transcriptase), integration of the reverse transcribed viral DNA into host cell chromosomes (5), transcription of viral genes from the resulting integrated provirus, translation of the transcribed viral sequences to produce viral proteins, including viral structural proteins required for virion formation, virion assembly at the membrane of the infected host cell with packaging of viral genomic RNA, budding of virions, with release of immature viral particles, and extracellular maturation of the virions, through cleavage of the viral *gag* protein mediated by the viral protease to yield mature, infectious virions (6). Additional steps in the replication cycle are being targeted in preclinical research in progress.

As of 2012, there were 30 different drug preparations approved by the US FDA for the treatment of HIV infection, including 23 distinct active pharmaceutical ingredients from seven different classes, acting via six different targets, including several fixed dose multidrug combination formulations (reviewed at http://www.fda.gov/ForConsumers/byAudience/ForPatientAdvocates/HIVandAIDSActivities/ucm118915.htm).

Use of anti-HIV drugs in nonhuman primate models of HIV infection and AIDS

Experience with the use of these drugs in the prevention or treatment of AIDS virus infection in NHP models has recently been reviewed (Del Prete & Lifson, 2013). The mainstay of treatment in these models has been the use of the nucleoside analog reverse transcriptase inhibitors (NRTI) tenofovir and emtricitabine, typically given as a daily subcutaneous injection, along with more variable regimen components comprised of oral or subcutaneously administered integrase strand transfer inhibitors (IN-STI) and/or protease inhibitors (PI), with occasional use of co-receptor blockers. While there has been considerable success in the use of anti-HIV drugs for the prevention and treatment of infection in such models, the cumulative experience has also identified some areas which indicate that direct extrapolation from human clinical experience may fail to identify specific challenges inherent in attempting to use these drugs in retrovirally infected animals of other species. There may be significant differences even between macaque species. Some of these challenges are outlined below.

Activity/Potency. Because of species specific viral restriction factors that limit the replication of HIV in NHP cells, most NHP models of HIV infection do not use HIV, but instead use various isolates of the related Simian Immunodeficiency Virus (SIV) or laboratory created chimeric viruses (Bieniasz, 2012; Hatziioannou & Evans, 2012). Many anti-HIV drugs are active against these simian viruses, but this cannot be assumed. For example, although many drugs in the NRTI class work well against simian viruses, their potency against simian viruses may be less than against HIV, and drugs of the non-nucleoside RT inhibitor (NNRTI) class, which act via a different mechanism than NRTIs to inhibit reverse transcriptase (RT), show virtually no activity against the RTs of the simian viruses. Some drugs, such as fusion inhibitors or co-receptor blockers, whose activity is specific to sequences in the HIV viral envelope glycoprotein or the co-receptors used by HIV, would not be expected to have activity against viruses having significantly different envelope glycoprotein sequences and using different receptor systems to gain access to target cells. Even for drugs that are active against simian viruses, the activity of other mechanistic classes of anti-HIV drugs, such as PIs and IN- STIs, is typically less against SIV enzyme targets than against the corresponding HIV enzymes they were developed to inhibit. Thus, despite the potent anti-HIV activity of many different antiretroviral drugs, their activity against other viruses in nonhuman species should not be assumed but must be empirically validated, in suitable in vitro assays, and ultimately in vivo. This is especially true for drugs that require intracellular metabolic activation for pharmacologic activity, such as the intracellular phosphorylation of NRTIs to their phosphorylated pharmacologically active forms. Ideally, in vitro testing should be done using cells of the relevant species, as such metabolic activation may vary between different target cells particularly if derived from different species.

Drug delivery. For sustained administration of ARVs to NHP, the two routes of administration that have been used most extensively are oral delivery and subcutaneous injection. For oral administration to NHP, drugs are generally mixed with food or dietary "treat" items. Challenges in this mode of administration include palatability/acceptance, compatibility of some drugs with different food items based on factors such as pH, the requirement to rotate the food item in which the drugs are presented to avoid boredom and eventual lack of acceptance, along with the relatively resource intensive requirements for staff time for preparation of the drug-in-food mixtures and monitoring to ensure complete consumption each dose (directly observed therapy), particularly for any medications that must be given more than once per day. Differences in oral bioavailability for different drugs, and between animals for the same drug are also important considerations, ideally addressed by monitoring blood levels. Oral absorption of drugs such as PIs and IN-STIs with poor aqueous solubility can be challenging. Many of these factors may be particularly challenging for administration of ARVs to koalas where the restricted dietary options may limit choices for oral administration of drugs, although anecdotal experience suggests that the IN-STI raltegravir can be effectively administered short term when given in eucalyptus flavored Portagen ® (C. Stadler, pers. comm.).

NHP can be behaviorally conditioned to accept subcutaneous injections, and for many drugs that are available in suitable formulations, this is a preferred method of administration. Compared to oral administration, subcutaneous administration is faster and more convenient, requires less staff time, and ensures full bioavailability. Important considerations include the volume to be injected, which in turn depends on the solubility of the drug(s) being injected, compatibility of the drugs included in multidrug combinations, and ensuring that the formulation does not induce any local injection site reactions, especially with sustained dosing. Daily subcutaneous administration of ARVs has been maintained for years in some NHP settings (Van Rompay et al., 2006, 2008, 2012). Work on development of long acting, sustained release formulations of anti-HIV drugs, including nanoformulated preparations, offers promise for more convenient dosing regimens in the future (Baert et al., 2009). Daily subcutaneous injections in koalas can be challenging, but anecdotal experience suggests that at least short term, daily administration of the NRTI tenofovir is feasible (C. Stadler, pers. comm.).

Pharmacokinetics. To maintain viral suppression, and avoid the selection of drug resistant mutant virus, it is important to maintain therapeutic levels of ARVs, particularly at the minimum concentration trough between doses ([C_{min}]). Drug levels are affected by absorption, and metabolism, and species differences in these parameters can affect pharmacokinetics, influencing drug levels over time. Indeed, even between different species of macaques, oral bioavailability of the same drug may vary. Drug metabolism may vary between species, and this may be particularly important for orally administered drugs such as many PIs and IN-STIs that in NHP require twice daily dosing to maintain therapeutic levels, typically defined as plasma levels in excess of the plasma adjusted IC_{95} for virus inhibition in vitro in a relevant assay, that is the drug concentration required for 95% inhibition of viral replication in the presence of plasma which contains proteins that can bind many drugs.

Administration of ARVs to koalas is complicated for orally administered agents by the restricted dietary options for this species and potentially by differences in absorption from a gastrointestinal tract quite different than that of primates (Stupans, 2001). In addition, for both orally and subcutaneously administered drugs potential differences in metabolism between koalas and primates may impact drug levels, emphasizing the desirability of pharmacokinetic analysis of drug levels to empirically determine dosages and administration schedules.

Safety/Tolerance/Toxicity/Drug-Drug interactions. While ARVs are administered to millions of people who in general tolerate them well, toxicities have been clearly identified and well described, particularly for the more commonly used agents. In NHP studies, the ARV related toxicity that has been best established is renal toxicity associated with acute or chronic overdosage with tenofovir, characterized by increased blood urea nitrogen and creatinine, and hypophosphatemia, with histologic findings of acute tubular necrosis and bone pathology, findings similar to tenofovir related toxicities described in humans (Calza, 2012; Sanders-Beer et al., 2011; Van Rompay et al., 2006, 2008, 2012). When multiple drugs are administered concurrently, the potential for drug:drug interactions must be addressed, with the realization that due to differences in drug metabolism drug:drug interactions may vary between species.

Sustainability. An important consideration for long term therapy is the sustainability of treatment, with multiple factors contributing. These include long term tolerance of the administered drugs and mode of dosing, but also maintaining the resources long term to source and administer the drugs.

Relation of drug target/activity to pathogenetic mechanisms. While all of the above considerations are important in contemplating the potential use of ARVs in KoRV infected koalas, arguably the most important consideration is the relation of the target of the drug and its mechanism of action to the pathogenetic mechanisms underlying the disease manifestation of concern. With ARV treatment of HIV infected humans or SIV infected NHP, the available licensed drugs all act to block new rounds of infection with no effect on already infected cells. This approach is efficacious in HIV and SIV infection because of the nature of the pathogenesis mediated by these viruses. In untreated HIV or SIV infection, the majority of viral replication is derived from de novo infection of CD4+ T cells that have a short life span once infected ($T_{<1/2>}$ approximately 1 day) (Wei et al., 1995). Thus, blockade of new rounds of infection substantially reduces overall viral replication levels and the immune activation that is associated with pathogenesis, including loss of CD4+ T cells and disease progression. However, even maximally suppressive ARV treatment of HIV infected patients does not affect virus production from already infected cells or impact latently infected cells. Thus, even prolonged ARV treatment producing maximal suppression of viral replication does not cure HIV or SIV infection as virus persists in cell populations not susceptible to ARV drug suppression, providing a source for recrudescent virus and progressive infection if ARV treatment is stopped (Richman et al., 2009). This has engendered a search for novel strategies beyond ARVs to effect HIV eradication or functional cure (Richman et al., 2009).

The details of the pathogenetic mechanisms underlying hematolymphoid malignancies in KoRV infected koalas remain to be fully elucidated. However, based on precedent from malignancies associated with other gammaretoviruses, it is likely that the underlying pathogenesis, once malignant disease is established, does not rely on de novo infection of new uninfected cells, and that high levels of plasma viremia reflect virus production from already infected cells (Bolin & Levy, 2011). In this situation, ARVs that block new rounds of de novo infection are unlikely to impact viral replication or disease processes. Indeed, anecdotal experience suggests that short term treatment of a KoRV-A/KoRV-B coinfected koala with a combination of a NRTI and an IN-STI did not meaningfully impact plasma viremia levels (C. Stadler, pers. comm.). And ARVs will not be expected to have any impact on endogenized virus, although they may help limit spread to new target cells of infectious forms potentially produced from endogenized sequences.

Thus, the potential applicability of ARVs to KoRV infection may be limited to certain situations. For example, treatment of infected dams and joeys may prevent transmission or pathogenesis of exogenous infectious forms of KoRV, an application well established for HIV and SIV (Mofenson, 2003). It is also possible that early ARV treatment may limit the replication and spread of exogenous infectious forms, potentially preventing viral integrations that may result in malignant transformation of target cells through insertional mutagenesis. However, such treatment might need to be sustained for life, and this prospect is likely not feasible with current drugs and delivery methods.

Alternatives to ARVs for prevention of KoRV infection

If ARVs may have a limited role in combating KoRV infection, what other interventions may be useful? While it has proven extraordinarily challenging to develop effective vaccines for the prevention or control of infection with lentiviruses like HIV or SIV, this is not the case for gammaretroviruses, where efficacious vaccines for feline leukemia virus (FeLV) have been developed (Hoover et al., 1996). This suggests that vaccines for other gammaretroviruses like KoRV-A and KoRV- B should be feasible. Such vaccines should help prevent transmission of exogenous infectious forms of KoRV-B, and potentially provide protection from pathogenesis from infectious forms expressed from endogenized KoRV-A. While a variety of approaches have been employed to develop candidate vaccines against FeLV, an approach that takes advantage of conserved features in the nucleocapsid (NC) proteins of all true retroviruses may be useful in developing a KoRV vaccine. The zinc finger motif in the NC proteins of all true retroviruses is present in KoRV (Shojima et al., 2013; Thomas & Gorelick, 2007). Site directed mutagenesis studies in HIV, SIV, and other retroviruses have shown that maintenance of an intact, authentic retroviral zinc finger motif in NC is required for completion of the viral replication cycle and NC has been implicated as a critical participant in multiple steps of retroviral replication (Thomas & Gorelick, 2007). Chemical treatments that preferentially covalently modify the free sufhydryl groups of the retroviral zinc finger motif in viral NC proteins result in elimination of infectivity, while preserving structurally and functionally intact envelope glycoproteins on the surface of treated virions (Arthur et al., 1998; Rossio et al., 1998). Such chemically inactivated retroviral virions have been shown to be useful vaccine immunogens in other retrovirus systems, and may merit evaluation as a candidate KoRV vaccine (Lifson et al., 2004).

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Immunization with Envelope Proteins of the KoRV as a Basis for a Preventive Vaccine

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Abstract. The rapid spread of the koala retrovirus (KoRV) in Australia and in international zoos calls for effective counter measures. As is the case with the human immunodeficiency virus (HIV) epidemic, a preventive vaccine is urgently needed. Vaccines inducing neutralizing antibodies are a good way to prevent retrovirus infections. Although for HIV there is still no effective vaccine available, commercial vaccines protecting cats from disease caused by the feline leukemia virus (FeLV) already exist and have been proven effective. KoRV is a retrovirus more closely related to FeLV than to HIV. Immunizing different species (rats, goats, hamsters, guinea pigs, mice, cats) with the transmembrane (TM) and surface (SU) envelope proteins of FeLV, as well as of the porcine endogenous virus (PERV) we always obtained neutralizing antibodies. PERV is also closely related to the KoRV. Based on the immunization studies with the envelope proteins of FeLV and PERV, we cloned and expressed the corresponding envelope proteins of the KoRV and immunized goats and rats. In all cases we obtained antibodies neutralizing the KoRV. However this does not mean that neutralizing antibodies will be obtained when immunizing koalas *(Phascolarctos cinereus)* with the envelope proteins of the KoRV or immunizing pigs with the envelope proteins of PERV. Therefore, koalas should be immunized with KoRV envelope antigens to determine whether neutralizing antibodies are induced and if so, whether such antibodies are able to protect healthy animals from infection. Furthermore, whether immunization with these antigens has a therapeutic effect on animals already infected with KoRV should be investigated. If *Chlamydia* infection of koalas is an opportunistic infection made possible by KoRV-induced immunodeficiency, immunization against KoRV will also protect animals from *Chlamydia* infection.

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Infection of koalas with the KoRV, and infection of humans with HIV-1 leading to AIDS. Retroviruses have long been known to be capable of infecting new host species by transspecies transmission. Interest in this subject has been boosted by the finding that the human immunodeficiency viruses (HIV-1 and HIV-2) are the product of such a transspecies transmission (Gao 1994,1999) and by recent concerns over the potential transmission of PERVs after xenotransplantation of pig organs into humans (Denner & Tönjes, 2012). The koala retrovirus (KoRV) is the result of such a transspecies transmission which is even associated with endogenization of the virus into the germ line of the animals (Hanger *et al*., 2000; Denner & Young, 2013). The KoRV is closely related to the gibbon ape leukemia virus (GaLV), which however remained exogenous in gibbons (Hanger *et al*., 2000). Both are related to endogenous retroviruses of South Eastern Asian mice, (Martin *et al*., 1999) and bats, (Cui *et al*., 2012a,b) however the origin and the transmission routes are still unknown.

Retroviruses are known to induce tumors and immunodeficiencies and HIV is the most prominent retrovirus inducing an acquired immunodeficiency syndrome. Although HIV, a lenti(retro)virus, and the KoRV, a gammaretrovirus, are not closely related, the clinical picture of the syndrome

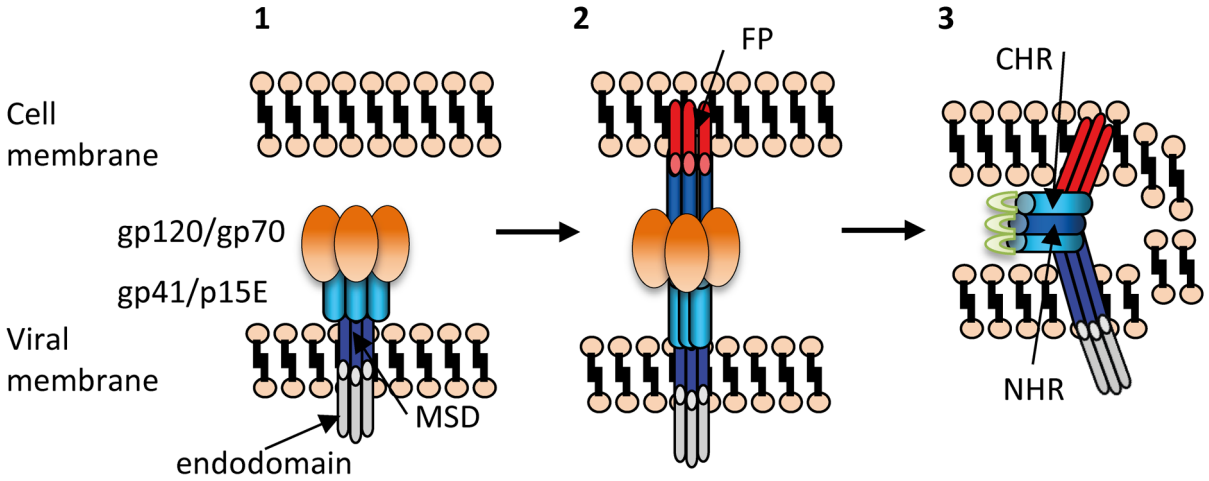

Figure 1. Schematic presentation of retroviral infection. Step 1: Interaction of the SU protein (orange, gp120, molecular weight 120,000 Dalton in the case of HIV-1; gp70 in the case of KoRV) with the cellular receptor (not shown). The TM protein (light blue, gp41, molecular weight 41,000 Dalton in the case of HIV-1, p15E, molecular weight 15,000 Dalton, E stands for envelope, in the case of the KoRV) is partially hidden in the SU protein, MSD, membrane spanning domain of the TM protein, dark blue. Step 2: Conformational changes in the TM protein, its fusion peptide (red, FP) penetrates the target cell membrane. Step 3: Interaction of the N-helical region (blue, NHR) and the C-helical region (light blue, CHR) of the TM protein and fusion of the viral and cellular membranes leading to subsequent internalization of the virus. Between the helical regions a hinge is shown composed of a Cys-Cys-loop (light green).

induced by HIV in humans and that induced by the KoRV in koalas *(Phascolarctos cinereus)* is similar concerning the immunodeficiency. HIV infections are usually accompanied by opportunistic infections among them *Chlamydia* infections (Contini, 2003). The major opportunistic infection in the case of the KoRV infection represents *Chlamydia* infection (Brown *et al*., 1987). *Chlamydia* infections are also commonly associated with FIV (feline immunodeficiency virus) infections (O'Dair *et al*., 1994). In addition, koalas infected with KoRV suffer from leukemia (Booth & Blanshard, 1999). Leukemia, lymphoma and immunodeficiency were also induced by FeLV which is closely related to the KoRV (Hardy 1985, 1993). Whereas only 5 to 10% of FeLV-infected cats suffer from leukemia and lymphoma, more than 65% of them die from opportunistic infection based on the immunodeficiency (Hardy 1985,1993). FeLV-infected cats as well as HIV-1-infected humans are characterized by a decrease in the number of $CD4^+$ cells (Hofmann-Lehmann *et al*., 1997). To summarize, a comparison of the KoRV infection with the infection with HIV-1 leading to AIDS may help to understand the immunopathogenesis.

Is vaccination more effective and economical than treatment?

Taking into account the costs of highly active antiretroviral therapy (HAART) used for the treatment of individuals infected with HIV-1 and the overall socio-economic impact of the AIDS pandemic on mankind, a vaccine protecting from HIV-1 infection would be the most efficient and cost effective of solutions. Unfortunately, such a vaccine is not yet available and until it is, the development costs for a vaccine depend on numerous factors. These include the selection of the best immunization strategy, the correct antigen and the most efficient adjuvant as well as the time and expense of preclinical and clinical trials. In the case of gammaretroviruses (to which KoRV belongs), the situation is quite different. For example, vaccines that protect from FeLV-induced disease in cats are commercially available and are being used successfully. In addition there are numerous publications demonstrating the efficacy of envelope antigens inducing neutralizing antibodies specific for other gammaretroviruses such as the FeLV, the PERV and different murine leukemia viruses (MuLV) (see below).

Neutralizing antibodies versus T cell immunity

There are two arms of the immune system, the humoral immunity based on B cells producing specific antibodies and the cellular immunity based on cytotoxic T cells (CTL). Most of the commercial vaccines protect humans from viral infection by inducing neutralizing antibodies. However, it is still unclear whether protection from retrovirus infections requires antibodies or CTL, or both. Retroviruses copy their genetic information, which is a single stranded RNA, into a double stranded DNA using the viral enzyme reverse transcriptase and later integrate this copy into the genome of the target cell. The DNA copy is the basis for the production of viral genomic and mRNA, of proteins and viral particles. On the other hand, the virus can persist undetected from the immune system if it does not express viral proteins. Therefore, neutralizing antibodies preventing infection in the first place represent the protection of choice. Neutralizing antibodies are usually directed against the envelope proteins which play an important role during infection (Fig. 1).

Neutralizing antibodies specific for the surface envelope protein gp120 and the TM protein gp41 of HIV-1 were found in HIV-1 infected individuals, however normally they cannot stop progression to AIDS (Kwong & Mascolla, 2012). Furthermore, some of these neutralizing antibodies were isolated, and generated as monoclonal antibodies. The localization of the epitopes recognized by these antibodies neutralizing HIV-1 is shown in Fig. 2.

Such monoclonal antibodies were shown to be broadly neutralizing, they inhibit infection with up to 90% of the HIV-1 strains (Muster *et al*., 1993; Zwick *et al*., 2001). Application of these human neutralizing antibodies to monkeys prevented an infection of the animals when they were challenged with infectious hybrid virus composed of the core of the simian immunodeficiency virus (SIV) and the envelope of HIV-1 (Mascola *et al.,* 1999; Ruprecht, 2009). Application of these broadly neutralizing antibodies to HIV-infected humans significantly decreased the virus load (Stiegler *et al*., 2002; Trkola *et al*., 2005). These data demonstrate that neutralizing antibodies are able to prevent a retrovirus infection *in vivo* and to inhibit progression to AIDS. However, until now such antibodies broadly neutralizing HIV-1 could not be induced in sufficient amounts after immunization with different envelope-derived antigens.

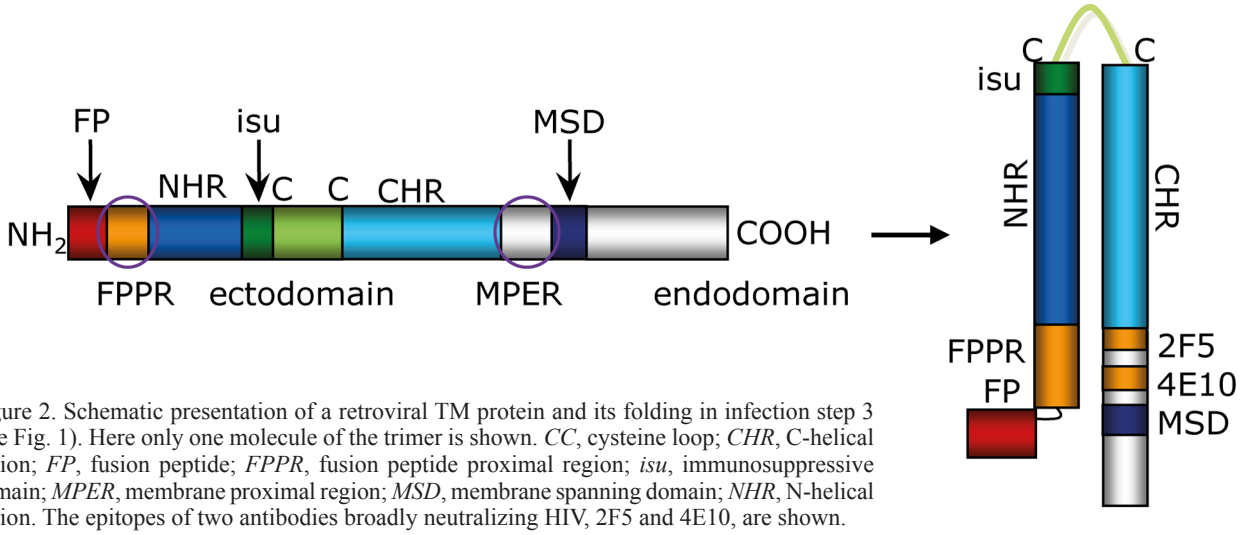

Figure 2. Schematic presentation of a retroviral TM protein and its folding in infection step 3 (see Fig. 1). Here only one molecule of the trimer is shown. *CC*, cysteine loop; *CHR*, C-helical region; *FP*, fusion peptide; *FPPR*, fusion peptide proximal region; *isu*, immunosuppressive domain; *MPER*, membrane proximal region; *MSD*, membrane spanning domain; *NHR*, N-helical region. The epitopes of two antibodies broadly neutralizing HIV, 2F5 and 4E10, are shown.

Neutralizing antibodies against MuLV, FeLV and PERV

In contrast to the non-successful attempts to induce neutralizing antibodies against HIV, antibodies neutralizing gammaretroviruses were induced easily. Many experiments have been conducted with potential murine leukemia virus vaccines. The approaches have included killed virus (Fink & Rauscher, 1964), subunit vaccines (Fischinger *et al*., 1976; Hunsmann *et al*., 1975; Hunsmann *et al*., 1981; Hunsmann, 1985), recombinant vaccinia viruses expressing viral gene products (Earl *et al*., 1986; Morrison *et al*. 1987), peptide vaccines (Bayer & Hunsman, 1987), and live attenuated viruses. Attenuation was achieved by prolonged passage through tissue culture (Mayyasi & Moloney, 1967; Ruan & Lilly, 1992), or by the use of live pathogenic virus blocked by antiretroviral drugs such as azidothymidin (AZT) and interferon alpha from replicating (Ruprecht *et al*., 1990, 1996). When mice were immunized with the SU (gp70, molecular weight 70,000 Dalton) and TM (p15E, molecular weight 15,000 Dalton, E stands for envelope) antigens of the murine leukemia virus (MuLV) substrain Friend leukemia virus (FLV) neutralizing antibodies were induced and protection from disease was reported (Fischinger *et al*., 1976; Hunsmann *et al*., 1975; Hunsmann, 1985; Schäfer *et al*., 1977; Thiel *et al*., 1987). Most importantly, the immune response and the protection were more efficient when both envelope proteins, p15E and gp70, were used for immunization. This was also true, when an immunotherapy was performed (Thiel *et al*., 1987). In AKR mice the onset of spontaneous leukemia induced by endogenous retroviruses could be dramatically delayed and the overall incidence was significantly reduced following treatment with high-titer heterologous antibodies against the surface envelope protein gp70 and p15E (Schäfer *et al*., 1976, 1977; Schwarz *et al*., 1976; Thiel *et al*. 1987; de Vos *et al*., 1998).

The mechanism of protection when immunizing with the envelope proteins was studied in transfer experiments. In one of these experiments mice were immunized with attenuated Rauscher leukemia virus (RLV), another substrain of MuLV. Passive transfer of the immune serum into mice challenged subsequently with infectious RLV was protective only at a very high serum dose, whereas immune T cells alone were fully protective, suggesting that cellular immunity alone is protective (Ruprecht *et al*., 1990, 1996). On the other hand, an essential role for virus-neutralizing antibodies in sterilizing immunity was described for Friend virus infection (Messer *et al*., 2004). In these investigations B cell-deficient mice were poorly protected by vaccination and passive transfer of neutralizing antibodies completely compensated for the B cell deficiency.

Similar immunization experiments were performed with envelope proteins derived from FeLV and first commercial vaccines were developed based on these immunizations (Pedersen *et al*. 1979; Pedersen, 1993; Pedersen & Johnson, 1991; Torres *et al*., 2010; Legendre *et al*., 1991). One of these commercial vaccines contains the recombinant SU envelope protein (Marciani *et al*. 1991). The SU protein in the virus is glycosylated (gp70), however the recombinant protein used for immunization was produced in bacteria and is not glycosylated, therefore its molecular weight is 52 kDa (recombinant, rp52).

We were mainly interested in using the TM protein of retroviruses for immunization (Denner, 2011, 2012). This interest was based on publications demonstrating that antibodies against the membrane proximal external region (MPER) of the TM protein gp41 of HIV-1 such as 2F5 and 4E10 (Fig. 2) isolated from HIV-infected individuals were neutralizing up to 90% of all HIV-1 (Muster *et al*. 1993; Zwick *et al*., 2001). We started to immunize with the TM protein p15E of PERV. Effective neutralizing antibodies were induced and epitopes in the MPER as well as in the fusion peptide proximal region (FPPR) were identified. The epitopes in the MPER of p15E were similarly located and despite the evolutionary distance between PERV and HIV-1 a sequence homology was observed. The epitope in the MPER sequence of gp41 of HIV-1 had the sequence NWFN/DIT, in the MPER of p15E of PERV the sequence GWFEGWFNRSP was recognized (identical amino acids are underlined) (Fiebig *et al*., 2003). Antibodies neutralizing PERV and binding to the FPPR and MPER were induced in different species including goats, rats, guinea pigs, hamster, rabbits, and mice (Fiebig *et al*., 2003; Kaulitz *et al*., 2011; Waechter *et al*., 2013). Using affinity chromatography and recombinant proteins corresponding to the N- and C-terminal part of p15E as well as synthetic peptides corresponding to the FPPR and MPER, we were able to show that only the isolated antibodies specific for the MPER were neutralizing (Waechter *et al*., 2013). When we immunized with a combination of the TM protein p15E and the SU protein gp70 (rp52) of PERV, higher titers of neutralizing antibodies were induced (Denner *et al*., 2012).

Since animal models are not available in which the efficacy of antibodies neutralizing PERV could be tested,

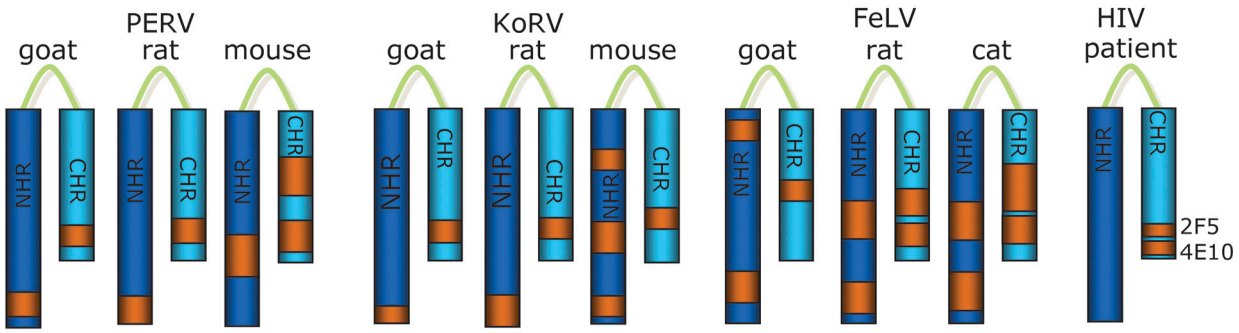

Figure 3. Schematic presentation of the epitopes recognized by neutralizing antisera induced in different species by immunization with the ectodomain of the transmembrane envelope proteins of PERV, KoRV, and FeLV. 2F5 and 4E10 were isolated from HIV-1 infected individuals and broadly neutralize HIV-1.

we used infections of cats with the related FeLV to study this topic. Immunization of cats (and several other species) with the TM protein p15E of FeLV resulted in neutralizing antibodies which recognized similar epitopes in the FPPR and MPER as described for PERV (FEGWFN in p15E of FeLV, HIV-1 and PERV see above, identical amino acids underlined) (Langhammer *et al*., 2005, 2006, 2011b). When we immunized with gp70 (rp52) of FeLV or a combination of both gp70 and p15E, the combination induced the highest titer of neutralizing antibodies (Langhammer *et al*., 2011a). When cats immunized with p15E, gp70 (rp52) and a combination of both were challenged with infectious FeLV, all animals immunized with gp70 (rp52) or the combination, and 50% of the animals immunized with p15E were protected from antigenemia and disease (Langhammer *et al*., 2011b). The absence of antigenemia indicates that the virus is not replicating and viral antigens cannot be detected in the serum. Thus, immunization with the envelope proteins protects the animals. However, even in the case of combination of both proteins, no sterilizing immunity was achieved (Langhammer *et al*., 2011b). Sterilizing immunity means complete protection from virus infection. In fact, protection from disease, but absence of sterilizing immunity was also reported for other commercial FeLV vaccines (Hofmann-Lehmann *et al*., 1997, 2007).

Envelope proteins of the KoRV induce neutralizing antibodies: basis for a vaccine

We had isolated a KoRV from an animal in the Zoo of Duisburg, Germany, which we designated KoRV Duisburg-Berlin ($KoRV_{D-B}$) (Fiebig *et al*., 2006). Part of the virus including the envelope proteins was sequenced (GenBank DQ174772). Only three amino acid substitutions in the Env region compared with a previously reported sequence of KoRV isolated in Australia were found (Hanger *et al*., 2000). We investigated the host range of the virus showing that the virus infected cells from humans and rats, but not from mice (Fiebig *et al*., 2006). These data were confirmed recently (Shojima *et al*., 2013). We characterized the protein pattern of purified virus and immunized with the recombinant TM protein p15E (Fiebig *et al*., 2006). p15E was cloned, expressed in *E. coli*, purified and used for immunization of goats, mice, and rats. A novel neutralization assay using $KoRV_{D-B}$ and susceptible human 293 cells was generated and we showed that the induced antibodies were neutralizing. The assay measures provirus DNA in the infected human 293 cells using real-time PCR (Fiebig *et al*., 2006). Epitope mapping showed that the sera recognized epitopes in the FPPR and MPER, and the sequence WFN was found in the MPER epitope (unpublished data) (Fig. 3).

Meanwhile we had also immunized with the purified SU protein gp70 (rp52) and with DNA corresponding to the Env protein gp70 and to the Env precursor molecule gp85. In all cases neutralizing antibodies were induced. The titer of neutralizing antibodies was higher when we immunized with gp70 compared with immunization with p15E (unpublished data).

Retroviruses cause immunosuppression

Many retroviruses induce immunosuppression in the infected host (Denner 1998, 2014; Mangeney *et al*., 2001; Mangeney *et al*., 2007; Oostendorp *et al*., 1993). Immunosuppression has been shown *in vivo* for HIV-1, HIV-2, MuLV, and FeLV and is always associated with opportunistic infections. The high prevalence of an opportunistic *Chlamydia* infection suggests that KoRV also induces immunosuppression. Unfortunately this has not been well-studied with *Chlamydia*, and, in addition, it is not known whether other opportunistic infections such as herpes virus and trypanosoma infection are increased in KoRV-infected animals. The mechanism how retroviruses induce immunodeficiencies is still unclear, but there is accumulating evidence that the TM protein is involved. We recently demonstrated that the TM protein gp41 of HIV-1 (Denner *et al*., 1994, 2013; Morozov *et al*., 2012), the TM protein of the human endogenous retrovirus HERV-K (Morozov *et al*., 2013) and the TM protein p15E of PERV (Denner, 1998; Tacke *et al*., 2000) inhibited lymphocyte activation by mitogens and modulated cytokine expression in PBMCs. The interleukins IL-10 and IL-6 were shown elevated and molecules involved in innate immunity were down regulated. When we studied purified KoRV, we showed that the virus particles induced enhanced expression of IL-10 in human donor PBMCs (Fiebig *et al*., 2006). Using a cytokine array, elevated expression of IL-10, of the grow-related oncogene GRO, of IL-6 and the monocyte chemotactic protein-1 (MCP-1) was observed after 24 hrs, whereas 18 other cytokines remained unchanged at that time (Denner et al, unpublished data). It was shown that all TM proteins contain a highly conserved domain, the so-called immunosuppressive (isu) domain (Fig. 2), and synthetic peptides corresponding to these domains are also able to inhibit lymphocyte activation and to modulate gene expression (Cianciolo *et al*., 1985; Denner *et al*., 1994; Ruegg *et al*., 1989).

We recently showed that single mutations in the immunosuppressive domain of gp41 of HIV-1 abrogated the immunosuppressive activity of the molecule and immunization with the mutated gp41 resulted in better antibody responses when compared with immunization with the wild-type gp41 (Morozov *et al*., 2012). It would be interesting to analyze whether mutations in the immunosuppressive domain of p15E of the KoRV also improves the immune response.

Conclusion and outlook

Koalas should be immunized with KoRV envelope antigens to determine whether neutralizing antibodies are induced and if so, whether such antibodies are able to protect animals from infection. Furthermore, whether immunization with these antigens has a therapeutic effect on animals already infected with KoRV should be investigated. Mutations in the immunosuppressive domain of the TM protein may increase the antibody response. Immunizing with a subunit of the TM protein of PERV we recently found novel neutralizing antibodies directed against an epitope in the N-terminal helix of the molecule (Denner & Waechter, 2014). Broadly neutralizing antibodies directed against the N-terminal helix of gp41 of HIV-1 were also found in HIV-infected individuals. Therefore a mixture of envelope antigens may be used for immunization. Prevention of infection or decreasing the virus load will prevent or reduce the potential KoRV-induced immunodeficiency and hopefully also protect koalas from infection with *Chlamydia* and other opportunistic infections.

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Potential Role of Human Restriction Factors in Inhibiting the Emergence of Koala Retrovirus (KoRV) as a Zoonotic Agent

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Abstract. The findings of an exogenous koala retrovirus (KoRV) associated with neoplastic diseases in koalas *(Phascolarctos cinereus)* brought up the concerns of infection by koala retroviruses in humans, especially koala handlers. As simple retroviruses, koala retroviruses lack the regulatory genes to counter restriction activities by human restriction factors in viral replication. Koala retroviruses belong to gammaretroviruses. Previous studies of susceptibility of murine leukemia virus and a lab contaminant retrovirus, gammaretroviral xenotropic MLV-related virus, to human restriction factors disprove the possibility of gammaretrovirus as a human pathogen. There is no evidence that the koala retrovirus can infect and replicate in human peripheral blood mononuclear cells, which is consistent with the resistant role of human restriction factors against gammaretroviruses.

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Retroviruses have existed and co-evolved with eukaryotic cells for millions of years. According to the genome organization, retroviruses can be divided into two broad groups, "simple" and "complex" viruses. Simple retroviruses contain only *gag, pol,* and *env* genes. Gammaretroviruses, including murine leukemia virus (MLV), feline leukemia virus (FeLV), gibbon ape leukemia virus (GALV), and koala retrovirus (KoRV) are simple viruses. Complex viruses contain regulatory/accessory genes in addition to their functional genes. A well-known example of a complex retrovirus is human immunodeficiency virus-1 (HIV-1) that contains two regulatory and four accessory genes; the latter appear to be dedicated to evade host defenses.

Mammalian cells have developed various innate self-defense mechanisms during the long battle to defend against infection by retroviruses. Among these anti-viral mechanisms three major classes of human restriction factors that block or restrict retroviral replication at different stages of life cycle have been described in detail through the studies of ecotropic MLV and HIV-1, including the APOBEC3 family of DNA cytidine deaminases, tripartite motif protein 5-alpha (TRIM5α), and tetherin (Malim, 2009; Wolf & Goff, 2008).

Three potent human restriction factors

The APOBEC3 restriction system comprises a family of polynucleotide cytidine deaminases. APOBEC3 proteins can be efficiently packaged into retroviral particles and inhibit replication by deaminating cytosine residues converting them to uracil during the first step of reverse transcription-the synthesis of minus strand DNA, which in turn, results in the guanine to adenine transition mutations in plus strand

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DNA in the infected cells (Malim, 2009; Wolf & Goff, 2008). The cytidine deaminase activity of human APOBEC3G and 3F can be neutralized by viral infectivity factor (Vif), an accessory protein of HIV, which can interact with APOBEC3 proteins and induce cellular proteasomal pathway to degrade these proteins (Marin *et al*., 2003; Mehle *et al*., 2004; Sheehy *et al*., 2003).

TRIM5α is a restriction factor first identified during the studies of the resistance to HIV-1 infection in old world monkeys (Stremlau *et al*., 2004), It belongs to the tripartite motif family, and contains a variable C-terminal SPRY or B30.2 domain that recognizes the capsid protein of an incoming retrovirus and determines the ability of TRIM5α to restrict specific retroviruses (Nisole *et al*., 2005; Perez-Caballero *et al*., 2005). TRIM5α inhibits infection subsequent to retroviral entry and delivery of the viral core into cytoplasm. It affects various retroviral core components and is proposed to cause premature disassembly and/or degradation of the reverse transcription complex, or block the nuclear translocation of the preintegration complex (Kutluay *et al*., 2013). The molecular mechanism of TRIM5α-mediated restriction is not fully understood. TRIM5α demonstrates some specificity in its restrictive capabilities. Human TRIM5α strongly inhibits MLV-N tropic and Equine Infectious Anemia Virus, but not MLV-B tropic, HIV-1 or Simian Immunodeficiency Retrovirus of Macaques (Keckesova *et al*., 2004; Perron *et al*., 2004).

Tetherin (previously known as HM1.24, BST-2 or CD317) was identified as a restriction factor through the study of HIV accessory protein Vpu (Neil *et al*., 2008). In the presence of tetherin, Vpu-minus HIV virions are assembled normally and adopt a normal morphology. However, large numbers of the mature virions remain trapped at the surface of infected cell membrane by tetherin, and some virions are subsequently internalized, leading to retention of viral particles both at the cell surface and within the endosomes of the infected cells (Neil *et al*., 2006; Perez-Caballero *et al*., 2009). The restrictive effect occurs solely at the stage of viral particle retention rather than assembly, and these "tethered" virions are fully infectious once released. It is not yet known how tetherin "tethers" virions to the cell surface, but its unusual topology may play a key role (McNatt *et al*., 2009; Perez-Caballero *et al*., 2009). The restriction of tetherin can be counteracted by the expression of Vpu in a not fully characterized cell type-specific manner (McNatt *et al*., 2009). Tetherins can block the release of a broad spectrum of retroviruses, ranging from alpharetrovirus, betaretrovirus, deltaretrovirus, lentivirus, to the spumaretrovirus genera of retroviradae (Jouvenet *et al*., 2009).

Susceptibility of gammaretroviruses to human restriction factors

Human restriction factors have been shown to inhibit replication of gammaretroviruses. Human APOBEC3G can restrict Moloney-MLV. Human TRIM5α strongly inhibits N tropic MLV, and tetherin potently blocks the release of MLV viral particles. In addition to MLV, the block of human restriction factors to gammaretroviruses was studied in detail through investigations of their effects on a gammaretroviral xenotropic MLV-related virus, XMRV. XMRV was first isolated from patients with familial prostate cancer, and then shown to be associated with chronic fatigue syndrome (Lombardi *et al*., 2009; Urisman *et al*., 2006). Although the link between XMRV and any human disease was disproven when XMRV was shown to be a lab-derived recombinant between two endogenous murine retroviruses (Cingoz *et al*., 2012; Delviks-Frankenberry *et al*., 2012), the research on the inhibition of XMRV by human restriction factors provides us with important insights on the barriers imposed on gammaretoviruses that prevent their assuming roles as human pathogens. APOBEC3 presumably inhibits XMRV replication when single round infectivity assays were used (Groom *et al*., 2010; Paprotka *et al*., 2010). XMRV replication can be restricted by tetherin but not by human TRIM5α (Groom *et al*., 2010). Human PBMCs express APOBEC3G and 3F, as a result XMRV can infect activated PBMCs, but with little or no replication and minimal spread (Chaipan *et al*., 2011; Groom *et al*., 2010). Hypermutation of XMRV provirus from infected PBMCs is reflective of the restriction mediated by APOBEC3 (Chaipan *et al*., 2011).

KoRV, like XMRV, does not encode a Vif-like protein to escape restriction imposed by APOBEC3 proteins. KoRV infection of human PBMCs has not been reported. The different variants of KoRV we have isolated from koalas housed in US zoos are not able to infect human PBMCs following exposure to cell-free virus, even though many human cell lines including T cell lines such as SupT1 and CEM are susceptible. Human restriction factors probably play a key role in the resistance of human PBMCs to KoRV and will most likely play a major role in restricting koala retroviruses from evolving into human pathogens.

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Leukemogenesis by Murine Leukemia Viruses: Lessons for Koala Retrovirus (KoRV)

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Abstract. Murine leukemia viruses (MuLVs) are the prototypical gammaretroviruses, and they have been extensively studied with regard to how they cause disease. Leukemogenesis by two MuLVs is reviewed here: the endogenous Akv MuLV of AKR mice, and exogenous Moloney MuLV. Important features of MuLV leukemogenesis include the in vivo generation of envelope recombinants (MCFs) through recombination with endogenous MuLVs, and induction of preleukemic changes typified by splenic hyperplasia secondary to bone marrow defects. Studies of MuLV leukemogenesis help to frame virological questions about how koala retrovirus (KoRV) may induce neoplastic or other diseases in koalas.

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The discovery of koala retrovirus (KoRV) in free-ranging and captive koalas *(Phascolarctos cinereus)* has been viewed with concern and interest. The primary concern is that KoRV-associated disease such as neoplasms, while yet to be conclusively proven to be KoRV caused, could increase the threats to survival of these animals. In the scientific community there is interest for several reasons: KoRV may be associated with lymphoma in koalas, it appears to be recently introduced into this species, and endogenization is an ongoing process. KoRV infection in koalas may provide an opportunity to study introduction and spread of a gammaretrovirus into a new host species and its accompanying effects. This process has happened in other species, notably mice, but in the more distant past, so some of the processes can only be deduced. At the same time, information learned from the relationship of murine gammaretroviruses and their hosts may provide lessons for understanding the potential relationships of KoRV and disease in koalas. The recent discovery of a second KoRV (KoRV-B) that may be associated with leukemogenicity (Xu *et al*., 2013) has similarities to oncogenesis in murine leukemia viruses (MuLVs). Leukemogenesis by MuLVs will be summarized here and possible implications to KoRV pathogenesis will be pointed out.

Murine leukemia viruses

MuLVs were first discovered in inbred mouse strains that had high incidences of leukemia. These studies resulted in isolation of several MuLV strains that cause leukemias of different hematopoietic lineages. For instance Moloney MuLV (M-MuLV) and Gross MuLV induce T-lymphoma, while Friend (F-MuLV) and Rauscher MuLV (R-MuLV) induce erythroleukemia and myeloid leukemia (Fan, 1997). These are the predominant MuLVs used in studies of MuLV leukemogenesis. They are prototypical retroviruses of the gammaretrovirus family.

MuLVs can be classified into types based on their envelope proteins and the kinds of cells that they infect, determined by the cell surface proteins that they bind. The leukemogenic MuLVs are mostly *ecotropic*; they infect cells of mice and rats, but they do not infect most

Table 1. Types of MuLVs according to host range.

virus class	susceptible cells	receptor name	function	examples
ecotropic	mouse, rat	CAT1	cationic amino acid transport	Akv-MuLV, Moloney MuLV
xenotropic	non-mouse	XPR1	phosphate export	xenotropic MuLVs
polytropic	mouse, non-mouse	XPR1	phosphate export	MCF MuLVs
amphotropic	mouse, non-mouse	PIT-2	phosphate import	amphotropic MuLV

other species. Their envelope proteins utilize the cationic amino acid transporter-1 (CAT1) molecule as the receptor (Table 1). Other MuLVs have been classified as *polytropic, xenotropic*, and *amphotropic*. Xenotropic MuLVs do not infect mouse cells, but they infect cells of other species. Polytropic MuLVs infect cells of both murine and non-murine origin; both xenotropic and polytropic MuLVs infect cells by interacting with the Xpr-1 molecule. Amphotropic MuLVs also infect mouse and non-mouse cells, but they infect by binding to the Pit-2 molecule.

Endogenous MuLVs

Endogenous retroviruses result from infection of germ cells; progeny resulting from these germ cells will genetically transmit the integrated viral DNAs as endogenous viruses (Jern & Coffin, 2008). Endogenization of retroviruses has occurred throughout evolution (millions of years ago in some cases), but it is an ongoing process in some species. Mice genetically transmit multiple copies of endogenous MuLVs, most of which cannot produce infectious virus (Stocking & Kozak, 2008). Nevertheless some of these defective endogenous viruses can be expressed and have biological effects, as will be described below. The endogenous MuLVs have been genetically mapped and classified according to their envelope types. In laboratory mice, the predominant endogenous MuLVs are derived from xenotropic and polytropic MuLVs (Table 2). The genetic loci containing these endogenous MuLVs have been designated *Xmv*s and *Pmv*s/*Mpmv*s respectively. Endogenous ecotropic MuLVs (encoded by *Emv*s) are present in some but not all laboratory mice.

Insertional activation of proto-oncogenes

A common mechanism for tumorigenesis by non-acute retroviruses (retroviruses that do not themselves carry an oncogene) is insertional activation of proto-oncogenes (Fan, 1997; Hayward *et al*., 1981). During retroviral replication, the viral RNA genome is reverse transcribed into viral DNA, which is integrated more or less randomly into the host cell DNA. During reverse transcription, long terminal repeats (LTRs) at either end of the viral DNA are generated; in the inserted (proviral) DNA form, the LTRs carry the signals for initiation of viral RNA synthesis by cellular RNA polymerase II (enhancers and promoters). A hallmark of non-acute retroviral oncogenesis is that independent tumors show proviral integration in common insertion sites (CISs). The CISs contain proto-oncogenes (normal cell genes involved in positive stimulation of cell division) that are transcriptionally activated by integration of the inserted provirus nearby. This can result from read-through transcription from the retroviral LTR (promoter insertion) (Hayward *et al.*, 1981), or by activation of the proto-oncogene promoter by the nearby viral enhancer in the LTR (enhancer activation) (Fan, 1997). Identification of CISs in retrovirus-induced tumors has led to identification of new proto-oncogenes (Cuypers *et al.*, 1984; Nusse & Varmus, 1982), some of which are also involved in human cancers. Oncogene discovery through identification of CISs in retrovirus-induced tumors is continuing today (Kool *et al.*, 2010; Suzuki *et al.*, 2002).

One consequence of the LTR activation of proto-oncogenes in non-acute retroviral oncogenesis is that the LTRs (and in particular the enhancers) influence both efficiency and type of disease. For instance, enhancer sequences are frequently duplicated in MuLV LTRs, and these duplications or other alterations increase both the transcriptional activities of the LTRs and also the rate at which the viruses induce leukemia (Lenz *et al.*, 1984). In addition, when M-MuLV and F-MuLV were compared, the disease specificity (T-lymphoid vs. erythryoid leukemia respectively) could be switched by exchange of the enhancer sequences (Li *et al.*, 1987). This was correlated with the fact that the M-MuLV LTR is preferentially active in T-lymphoid cells while the F-MuLV is preferentially active in erythroid cells (Short *et al.,* 1987). Since the LTR enhancers are important in LTR activation of proto-oncogenes it is logical that an MuLV will induce tumors of the cell type where its LTR enhancers are most active.

While insertional activation of proto-oncogenes is a fundamental mechanism for oncogenesis by non-acute retroviruses, it has also become clear that other virus-induced events are also important. This will be discussed in the context of two well-studied MuLV systems: endogenous Akv MuLV of AKR mice, and M-MuLV.

Table 2. Endogenous viruses of laboratory mice. (Classification according to host range of the Env protein. Most, but not all, endogenous proviruses cannot encode infectious virus; some defective proviruses can participate in recombination with exogenously infecting MuLVs [e.g., Pmv's and Mpmv's]).

class	genetic loci	comments
xenotropic	Xmv's	Xmv1 is readily activated in some mouse strains.
polytropic	Pmv's	Envelopes from both classes bind Xpr1 receptor; multiple copies of both classes are in most mice.
modified polytropic	Mpmv's	
ecotropic	Emv's	Relatively few or no copies in most mouse strains.

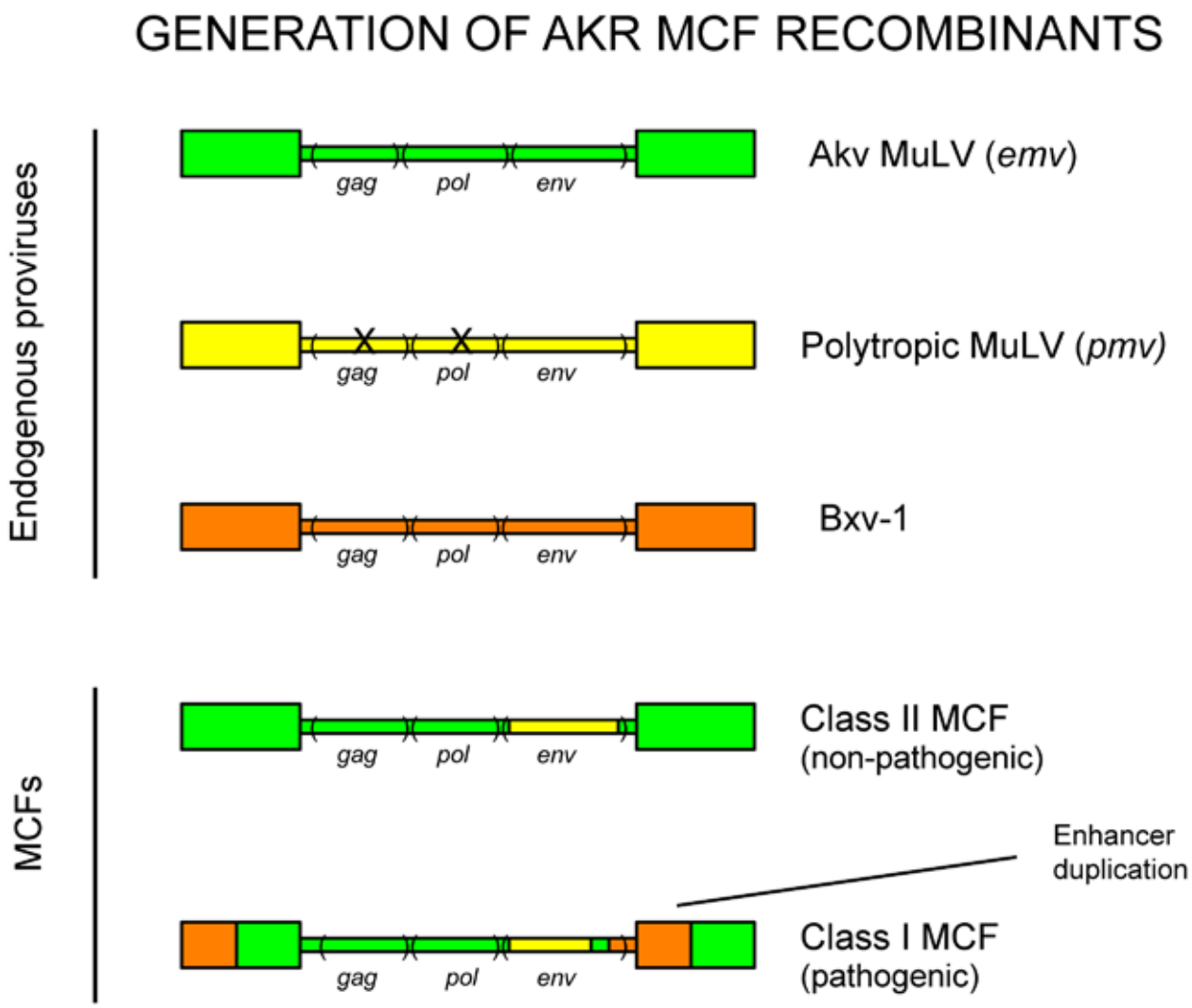

Figure 1. Generation of MCF recombinants in AKR mice. The organization of the endogenous proviruses that give rise to AKR MCFs is shown in the upper part of the figure. Akv-MuLV results from induction of one of two endogenous ecotropic proviruses (encoded by *Emv-10 or -12*). Recombination with a *Pmv or Mpmv* provirus gives MCF recombinants. The lower part of the figure shows class I and II MCFs; class II MCFs simply represent recombination between Akv-MuLV and an *Pmv* or *Mpmv* provirus, while class I MCFs result from additional recombination with *Bxv1* xenotropic endogenous virus.

Leukemogenesis in AKR mice

Inbred AKR mice develop T-cell lymphoma with a latency of 6–7 months. These mice genetically transmit two endogenous ecotropic MuLV proviruses (*Emv10* and *-12*) ; which both can encode replication-competent MuLV (termed Akv-MuLV). Akv-MuLV is activated in AKR mice after birth, and once activated it carries out additional rounds of infection in the animals. Activation of Akv-MuLV is required for leukemogenesis in AKR mice.

Hartley and Rowe made the seminal observation that AKR mice develop recombinant versions of Akv-MuLV close to the time when leukemia occurs (Hartley *et al.*, 1977). These recombinants result from recombination between Akv-MuLV and an endogenous polytropic virus, which results in the recombinant virus carrying polytropic envelope sequences in place of the Akv *env* sequences. (Fig 1) The resulting viruses were termed mink cell focus-inducing (MCF) recombinants because they cause cytopathic effect in vitro when infecting mink lung fibroblasts. AKR MCF recombinants infect cells by binding to the Xpr1 receptor instead of the mCAT1 receptor. The fact that MCF recombinants were detected in AKR mice close to the time that leukemia developed led Hartley and Rowe to propose that MCF recombinants are the "proximal leukemogens" in these mice (Hartley *et al.,* 1977).

Additional studies of AKR MCF recombinants revealed another layer of complexity. The AKR MCF recombinants arising in these mice could be further subdivided into Class I and Class II MCFs. The class I MCFs were considered pathogenic because they could accelerate lymphomagenesis when infecting AKR mice; on the other hand the class II MCFs were not pathogenic by this acceleration assay (Holland *et al.*, 1985). Molecular analysis revealed that class II MCFs are recombinants containing an endogenous polytropic MuLV envelope, while class I MCFs actually result from two recombinations (Stoye *et al.*, 1991). In class I MCFs, the envelope sequences are polytropic, but additional recombination with another endogenous virus (the xenotropic *Bxv-1* provirus) results in the LTR and its enhancer sequences being derived from *Bxv-1* (Fig 1). The higher activity of the *Bxv-1* LTR compared to the Akv-MuLV LTR is thought to result in the pathogenicity of the class I MCFs.

There are several possible mechanisms by which class I MCFs contribute to leukemogenesis in AKR mice. First MCF recombinants would allow continued infection in animals where the majority of cells are already infected with Akv-MuLV. Cells infected by a retrovirus exhibit resistance

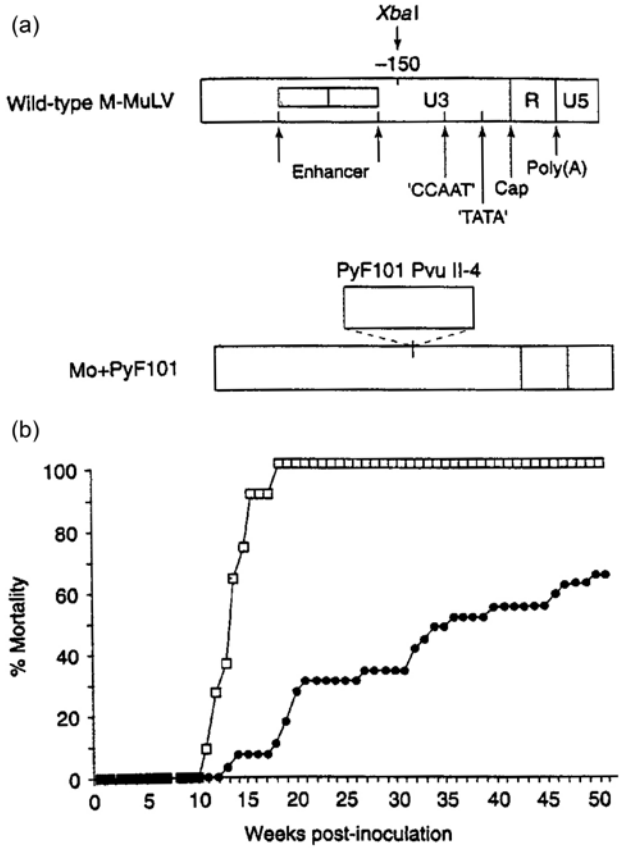

Figure 2. The Mo+PyF101 variant of M-MuLV. *(a)* The organization of the wt M-MuLV LTR is shown at the top; the location of the inserted PyF101 enhancers is shown below. *(b)* NIH Swiss mice were inoculated subcutaneously with wt or Mo+PyF101 M-MuLV. The per cent mortality from T-cell leukemia is shown as a function of time. Filled symbols represent animals infected with Mo+PyF101 M-MuLV and open symbols represent animals infected with wt virus.

to superinfection by viruses with envelopes that bind to the same cellular receptor, but they can be infected by viruses that utilize a different receptor. Thus, in an AKR mouse, cells infected by Akv-MuLV could be re-infected by an MCF. Second, since the range of cells in an AKR mouse that Akv-MuLV infects is determined by those cells expressing the ecotropic mCAT1 receptor, MCF recombinants could potentially infect additional cell types that express the Xpr1 receptor but not mCAT1. Third, MCFs could have physiological effects that contribute to tumorigenesis. It has been reported that MCF envelopes bind to cellular growth factor receptors such as the interleukin-2 receptor (IL-2R), leading to factor-independent growth of IL-2 expressing T-lymphocytes (Li & Baltimore, 1991). Other studies have indicated that MCFs lead to premature thymic atrophy resulting from lysis of infected thymocytes (Haran-Ghera *et al*., 1987). This could lead to repopulation of the thymus with cells with leukemic potential ("preleukemic cells").

A final point should be made about AKR leukemogenesis. While the appearance of class I MCFs is associated temporally with the development of leukemia, and infection of AKR mice with a class I MCF accelerates the rate of disease, class I MCFs do not cause leukemia when used to infect mice that are not infected with an ecotropic MuLV. Thus the rapid leukemia in AKR mice appears to result from infection by both ecotropic Akv-MuLV and a class I MCF.

Leukemogenesis by M-MuLV

M-MuLV induces T-cell lymphoma in 3–4 months when inoculated into newborn mice (Fan, 1997). M-MuLV-induced leukemias show proviral activations of cellular proto-oncogenes such as *c-myc* and *pim-1* and a variety of others. M-MuLV-infected mice also generate MCF recombinants, although M-MCFs retain the M-MuLV LTR likely because it is highly active in T-lymphoid cells. Like AKR MCFs, M-MCFs do not efficiently induce disease when inoculated into mice in the absence of M-MuLV. Thus many of the virological principles for leukemogenesis in mice infected with exogenous M-MuLV are shared with spontaneous leukemia in AKR mice.

We have studied M-MuLV leukemogenesis, using a virus with a modified LTR, Mo+PyF101 M-MuLV. This virus has enhancer sequences from the F101 variant of murine polyomavirus inserted into the M-MuLV LTR between its enhancer and promoter (Fig. 2) (Linney *et al.*, 1984). When inoculated subcutaneously into NIH Swiss mice, Mo+PyF101 M-MuLV shows reduced leukemogenicity (Davis *et al.*, 1985). Comparative studies revealed a virus-induced preleukemic state induced by wt but not Mo+PyF101 M-MuLV, typified by mild splenomegaly and hyperplasia of multiple hematopoietic lineages (Davis *et al.*, 1987). Thus this preleukemic hyperplasia was correlated with the efficient induction of leukemia by wt but not Mo+PyF101 M-MuLV. Further studies indicated that the splenic hyperplasia was secondary to a virus-induced defect in bone marrow hematopoiesis, and the reduced leukemogenicity of Mo+PyF101 M-MuLV was correlated with the absence of the bone marrow defect (Li & Fan, 1991). These effects are reminiscent of myelodysplastic syndrome in humans, where defects in bone marrow hematopoiesis lead to compensatory extramedullary hematopoesis (e.g., splenic hyperplasia) and increased incidence of leukemias.

An explanation for the bone marrow defect and splenic hyperplasia was provided by the observation that mice inoculated subcutaneously with Mo+PyF101 M-MuLV do not generate MCF recombinants (Brightman *et al.*, 1991). Moreover, infection of NIH-3T3 fibroblasts or primary mouse bone marrow cultures with a combination of M-MuLV and M-MCF was growth inhibitory, while infection with either virus alone did not inhibit cell growth (Li & Fan, 1990). These results indicate a role for M-MCF recombinants early in the disease process, i.e. induction of the preleukemic state, although they do not exclude involvement of MCFs in later stages of M-MuLV leukemogenesis. The roles of M-MuLV and M-MCFs in multiple steps of leukemogenesis are shown in Fig 3. In addition to the early events described, re-infection of M-MuLV induced T-lymphomas and activation of proto-oncogenes in tumor progression (tumor progression loci) has been documented (Bear *et al*., 1989; Gilks *et al.*, 1993).

In summary murine leukemia viruses not only activate proto-oncogenes during leukemogenesis, but they induce changes that promote development of tumors both during preleukemic phases, and also during tumor progression. Envelope recombinants (MCFs) are involved in some of these processes.

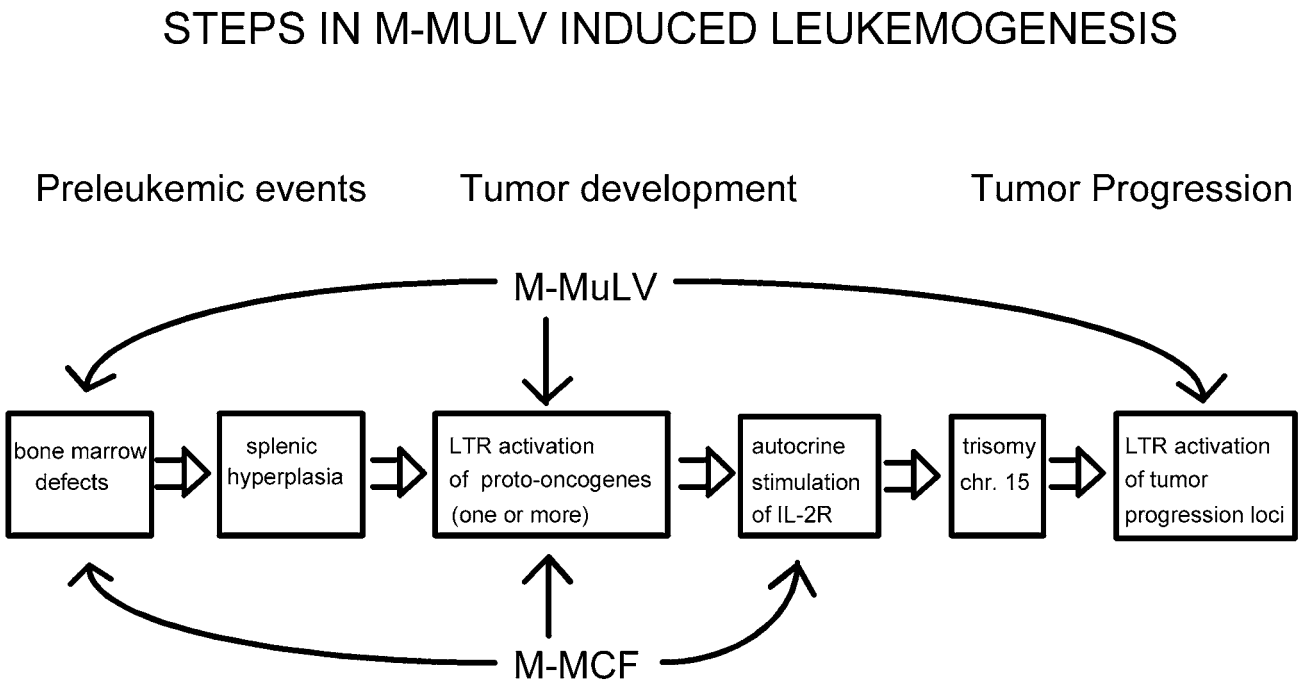

Figure 3. Steps in M-MuLV-induced leukemia. Different steps in development of leukemia after M-MuLV infection are shown, and the roles of M-MuLV or MCF recombinants are indicated.

Lessons and perspectives from MuLVs on potential KoRV leukemogenesis

As discussed elsewhere in this volume, the high incidence of lymphoma in koalas along with other neoplastic or pre-neoplastic conditions is highly suggestive of KoRV causing some of these diseases, particularly the lymphomas. This is supported by the close evolutionary relatedness of KoRV and MuLV, and the similarity of the koala diseases to MuLV-induced diseases. However, definitive proof that KoRV is inducing leukemia in koalas (e.g., integrated KoRV DNA at CISs in tumors) has not been reported. Hopefully ongoing investigations will provide such proof.

The recent description of a second KoRV strain, KoRV-B that may be associated with enhanced leukemogenicity is particularly noteworthy (Xu *et al.*, 2013). KoRV-B differs from the original KoRV (A) sequence by having an envelope protein that recognizes a different cellular receptor (the thiamine transporter vs. Pit-1), and it also has a triplication in the enhancer motif in the LTR. These differences are quite reminiscent of pathogenic Class I AKR-MCFs, which contain polytropic Env as well as altered LTR enhancers from the Bxv-1 endogenous virus. Other investigators have obtained evidence for other Env region variants of KoRV in infected animals (Shojima *et al., 2*013) (P. Young, unpublished), but it is not yet clear if these Env variants bind to different cellular receptors, and how frequently they are observed. The origins of the new Env sequences in KoRV-B and the other new variants is also interesting. Do these viruses represent recombination with endogenous KoRV-related proviruses, analogous to the contributions of endogenous MuLVs to generation of MCFs? Alternatively, do they represent recombination with other enveloped viruses? Ongoing sequencing of the koala genome should provide insight into these questions.

By analogy to MuLV leukemogenesis, if a causative role for KoRV in development of lymphoma or other neoplasms is confirmed, it may be useful to consider KoRV-A as analogous to ecotropic Akv-MuLV or M-MuLV, while KoRV-B might be analgous to an MCF recombinant. In this light the following questions can be asked:

1 Is KoRV-A by itself able to induce leukemias, and if so how efficiently?

2 Is formation of Env recombinants (KoRV-B and others) a common feature of KoRV-A infection or leukemogenesis in koalas?

3 Is KoRV-B capable of inducing leukemia by itself, or is co-infection with KoRV-A required?

4 Are some of the hematopathologies in KoRV-infected koalas analogous to preleukemic changes in M-MuLV-infected mice (bone marrow dysplasia, splenic hyperplasia)?

While it may be difficult or impossible to address these questions experimentally, in any event considering them conceptually will help to clarify virological aspects of KoRV leukemogenesis.

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KoRV and *Chlamydia*: Are they Co-culprits?

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Abstract. There are two main infectious disease threats for the koala; *Chlamydia* and KoRV. A major question is whether or not KoRV predisposes koalas to more severe chlamydial disease. In the only study to date that has examined co-infections, KoRV load (as determined by qPCR) and chlamydial load (as determined by qPCR) and chlamydial disease were examined in wild koalas. While there was a statistically significant correlation between *Chlamydia* infection load and *Chlamydia* clinical disease score, there was no significant correlation between KoRV load and either *Chlamydia* infection load or *Chlamydia* clinical disease score, however the groups were not ideally constructed and hence additional comparisons are needed. If KoRV does predispose koalas to more severe chlamydial disease, one would expect it to do this via an effect on the koala immune system. A series of *Chlamydia* vaccine trials in captive as well as wild koalas are showing that koalas in fact appear to make perfectly normal antibody and cytokine responses to vaccine antigens, even if they have high circulating KoRV loads, arguing against an immune suppressive effect by KoRV.

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In Australia, wild koala (*Phascolarctos cinereus*) populations in many areas, particularly Queensland and NSW, are declining for many reasons. One of the main causes of these declines is infection and disease due to *Chlamydia* (Polkinghorne et al., 2013). While *Chlamydia* cause similar disease syndromes in their non-koala hosts, the koala seems to have a higher than expected level of disease. This raises the question as to whether or not KoRV is somehow contributing to chlamydial disease. This brief overview will focus first on what we know about *Chlamydia* in koalas and then look at the very limited data regarding KoRV and *Chlamydia*.

Overview of *Chlamydia*

Chlamydia is an obligate intracellular bacterium with a unique two-phase developmental cycle. Immunity to chlamydial infections requires both a strong, neutralising antibody response as well as an interferon-gamma directed T cell response. Of the nine species present in the genus *Chlamydia*, two, *C. pecorum* and *C. pneumoniae*, cause infections in koalas (Jackson *et al*., 1999; Deveraux *et al*., 2003). The frequency of chlamydial infections (measured by a range of techniques, but utilizing PCR mostly of late) varies between populations, ranging from nil (on just a few island populations) to 90% in several populations (Polkinghorne *et al*., 2013). Disease levels also vary, but usually represent 25% or so of the infection level at any time point sampled. Animals are infected at ocular and urogenital sites mainly. Of the two chlamydial species, *C. pecorum* is by far the most common and is thought to be the species responsible for the symptoms observed (Glassick *et al*., 1996).

Even though it is *C. pecorum* that is responsible for most infection and disease in koalas, there is considerable genetic diversity between sub-strains (Jackson *et al*., 1997). A range of gene markers have been used to assess *C. pecorum* strain diversity and while there are some minor differences, they all show that the various koala *C. pecorum* genotypes cluster together, but show considerable strain diversity.

Chlamydia infection and clinical disease

A key question that is still unanswered is how chlamydial infection or strain variation, relates to overt clinical disease. We know from other species of *Chlamydia* that different strains and sub-strains account for differences seen in pathogenicity (although this is still a new area of research for all *Chlamydia*). Therefore, given that we have considerable strain diversity within the strains of *C. pecorum* infecting koalas, it is also quite conceivable that this strain diversity also explains the virulence difference observed.

Chlamydia and KoRV

Finally, is there any evidence linking KoRV infection to adverse pathogenicity for chlamydial co-infections ? There is very limited data on which to make any comments. The Queensland University of Technology (QUT) group is developing an anti-*Chlamydia* vaccine and this has been used to vaccinate a significant number of koalas now. In several of these trials, in a captive koala colony, the vaccinated animals were tested for exogenous KoRV levels and found to have high KoRV copies per ul (greater that 10^6 and even up to 10^8). Despite these high levels of exogenous KoRV, all animals produced a very strong B and T cell response to the chlamydial vaccine.

The only other study was conducted as a collaboration between researchers at University of Queensland and QUT. Log KoRV load (measured as exogenous KoRV via PCR) was analysed against *Chlamydia* infection load (as measured by a quantitative PCR assay) and *Chlamydia* disease score. While there was a statistically significant correlation between *Chlamydia* infection load and *Chlamydia* clinical disease score, there was no significant correlation between KoRV load and either *Chlamydia* infection load or *Chlamydia* clinical disease score. This study however did have several limitations and deserves to be repeated.

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The Koala Genome Consortium

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Abstract. The koala *(Phascolarctos cinereus)* is an iconic Australian animal. Koalas are both biologically unique and evolutionarily distinct as the only living representative of the marsupial family Phascolarctidae. Their unique and highly specific diet of eucalyptus leaves, combined with the increasing threats of predation and habitat loss through urbanisation, mean that koalas are particularly vulnerable to the deleterious effects of fragmented habitat and population bottlenecks. They are further threatened by disease such as *Chlamydia* and there is increasing interest in the varying strains of the Koala Retrovirus. We present preliminary transcriptome and genome data for the koala and introduce the Koala Genome Consortium (KGC), a group working towards the production of a high quality draft assembly of the koala genome. The KGC is currently comprised of several Australian research institutes and Universities although our intention is to recruit researchers from around the world to contribute to the genome assembly and annotation process and ultimately make use of the assembled genome. Once available as an annotated draft, we anticipate the genome sequence will add significant value to the extensive body of existing research for koalas.

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Overview of the Koala Genome Consortium

The purpose of the Koala Genome Consortium is to generate a high quality draft assembly of the koala genome, which will be useful to all koala researchers and have real and measurable conservation outcomes for koalas. Prior to the sequencing of the koala genome, we also undertook to determine the transcriptome from several tissues from two separate koalas. The Koala Genome Consortium is a project co-led by the Australian Museum and Queensland University of Technology but since this is a large undertaking, demanding the expertise of a wide group of researchers in the fields of koala biology, bioinformatics and marsupial genomics, we are currently seeking expressions of interest from potential new Consortium members and will continue to do so over the course of the project.

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Koala genome

We obtained tissue samples from a female koala named Pacific Chocolate from Port Macquarie Koala Hospital in New South Wales, and high molecular weight gDNA extractions from liver tissue were used to prepare libraries suitable for massively parallel sequencing using the Illumina platform. Our initial genomic sequencing datasets represent approximately 100-fold coverage of the genome with the large task ahead to assemble and annotate the genome. It is our intention that the formation of the Koala Genome Consortium (www.koalagenome.org) will facilitate new collaborations between all groups with a scientific interest in the koala genome data, thereby producing a comprehensive and well annotated assembly which will benefit the already substantial efforts dedicated to koala research globally.

Koala transcriptome

We have generated koala transcriptome data from eight different tissues from two separate koalas, *(a)* Pacific Chocolate, from the Port Macquarie region of NSW, and *(b)* Birke, from the South East region of Queensland. We chose multiple tissues to allow preliminary comparison between gene expression in different koala tissue types (i.e. brain, heart, lung, liver etc). Our transcripts are now fully assembled and are undergoing annotation. We have sequenced our transcripts to a very high level of coverage, estimated to be ×100 fold, which we anticipate will enable us to detect genes with low expression levels.

Why is the Australian Museum so interested in a genome sequencing project?

Our own research focus is largely around population and evolutionary genetics, as well as utilizing our historic natural history collections. The Australian Museum is not only the oldest museum in Australia but is one of the oldest in the world, and has extensive natural history collections of Australia's most iconic animals dating from present day back to the mid 1850's. Museum collections have already been used to give insight into historic KoRV infections and give insight into historic levels of mitochondrial diversity (Ávila-Arcos *et al*., 2012; Tsangaras *et al*., 2012). Further, the Australian Museum is periodically approached for population management advice based on population genetic data, including for NSW koalas for which we have developed additional microsatellite markers using next generation sequencing and the KGC data. One of our priority research outcomes is to develop and implement a suite of highly variable SNP markers that can be used for direct management outcomes in addition to the microsatellite data.

The Australian Museum team is especially interested in using the genome and transcriptome data to understand the koala's unique physiological adaptations and in particular, it may be possible to predict response to environmental change to develop strategies to mitigate damage to koala populations in the longer term.

The koala genome and koala retrovirus (KoRV)

With a high quality, deep coverage whole genome and transcriptome assembly from two animals we can ascertain how and where the different strains of KoRV have been endogenized in the koala. By comparing multiple tissue types and two animals initially we will be able to determine if two animals share insertion sites, or if they are more likely to be individually variable. This represents a significant advance in what is currently known about KoRV and will be of benefit to the wider research community.

Get involved in the project via Koalagenome.org

We have established www.koalagenome.org as a focal communication, data-sharing and data-storage point for the Koala Genome Consortium. Please contact us at this site or via our email address: koalagenome@austmus.gov.au if you have an interest in being involved with the project.

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Anti-retroviral Drugs and Vaccines

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Abstract. This manuscript summarizes the break-out session held on anti-retroviral drugs and vaccines at the *Koala Conservation Workshop: The koala and its retroviruses: implications for sustainability and survival* held at San Diego Zoo, April 17–18, 2013. Discussants considered the utility of natural retroviral systems as models for treatment and prevention of koala retrovirus (KoRV) infection, in particular, feline leukemia virus infection of the cat and AIDS virus infections of humans and non-human primates. Key lessons learned from those model systems may be applicable to the development of anti-retroviral drugs for treatment of KoRV infection or vaccines to prevent it. Aspects of the experience with model systems that are most likely to be translatable to KoRV infection include the identification of optimal drug targets, parameters for drug delivery, components of an effective vaccine, and approaches to measure protection.

Levy, Laura S., and Jeffrey D. Lifson. 2014. Anti-retroviral drugs and vaccines. In *The Koala and its Retroviruses: Implications for Sustainability and Survival*, ed. Geoffrey W. Pye, Rebecca N. Johnson and Alex D. Greenwood. *Technical Reports of the Australian Museum, Online* 24: 93–95.

1 What natural retroviral systems might serve as useful models for treatment and prevention of KoRV infection?

FeLV infection in the cat

- Both FeLV and KoRV infect the natural host in the wild.
- Natural infection is associated with leukemia/lymphoma and with wasting disease, among other less common disease outcomes.
- Natural infection is frequently cleared by an effective immune response.
- Endogenous and exogenous viruses occur in both systems.
- FeLV and KoRV occur naturally in distinct subtypes defined by envelope sequence and receptor utilization. They utilize common receptors.
- FeLV-A is the horizontally transmitted subtype spread cat-to-cat in nature. Other subtypes arise *de novo* in each infected animal. The horizontally-transmissible FeLV-A subtype appears to be analogous to KoRV-B.

AIDS virus infections in nonhuman primates (NHP)

- AIDS viruses are lentiviruses (HIV, SIV) rather than gammaretroviruses (KoRV-A,B; FeLV-A,B).
- SIV infection occurs in the natural host (African NHP) and in experimental infection of Asian macaques.
- The virus is exogenous. Typically horizontal transmission occurs although vertical transmission (maternal-fetal or via nursing) can occur.
- Infection of natural hosts does not typically lead to progressive disease.

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- Experimental infection of Asian macaques results in progressive immunodeficiency and disease/death usually through opportunistic infections and neoplasms.

2 Possibilities for treatment of KoRV infection

What key lessons have we learned from anti-retroviral drug development that may be applicable to treatment of KoRV infection?

Drug treatment for retroviral infection is an actuality. As of 2012, the FDA has approved 30 anti-HIV drugs of seven different classes with six different viral targets.

What might be the optimal targets for anti-KoRV therapeutics?

- Anti-HIV drugs have been developed to target viral enzymes, including reverse transcriptase, integrase and protease.
- Anti-HIV drugs have been developed to target viral envelope/receptor interactions and block virus entry.
- Although antiretroviral drugs have been developed based on activity against HIV, some of them are also active against SIV and other retroviruses.

What is the nature and basis of the pathogenesis to be targeted with anti-KoRV drug development?

- For appropriate drug targeting, the source of virus production and the mechanistic basis of virus-induced pathogenesis in the infected animal must be identified.
- Available antiretroviral drugs block new rounds of *de novo* infection but do NOT impact cells that are already infected.

What are the optimal parameters for anti-KoRV drug delivery?

- Parameters to be examined include activity/potency against KoRVs, dosing, route, pharmacokinetics and drug metabolism, bioavailability and toxicity.
- The koala gastrointestinal tract is different than human or NHP; oral drug absorption, bioavailability, metabolism, pharmacokinetics need to be determined empirically.
- Subcutaneous delivery is more reliable, but less practical for daily administration. Metabolism and pharmacokinetics issues remain.

Potential limitations of anti-KoRV drug treatment?

Available antiretroviral drugs block new infection, but would not target a self-renewing population of already infected malignant transformed cells with integrated provirus. In the case that such cells represent the source of virus in KoRV-infected animals, then antiretroviral drugs may not be an effective approach.

3 Possibilities for prevention of KoRV infection

What key lessons have we learned from FeLV and HIV/SIV vaccine development that may be applicable to development of a KoRV vaccine?

- FeLV vaccination has been largely successful and serves as a useful model for the potential of KoRV vaccination.
- The frequent clearance of natural FeLV infection by an effective immune response was a key initial indicator that vaccination might be successful.
- Determinants of effective FeLV immunity include virus neutralizing antibody and active cell-mediated immune response, although the correlates of protection remain incompletely understood.
- Protection is elicited when FeLV subgroup A is included in the vaccine.
- FeLV vaccination is highly protective but does not induce sterilizing immunity and no currently available FeLV vaccine provides 100% protection. Vaccinated animals typically clear the virus from the blood after challenge, but residual proviral DNA and viral RNA can be detected in blood and bone marrow.
- For HIV/SIV, examples of spontaneous clearance of infection are rare to non-existent and the nature of clearly protective vaccine-induced immune responses remains to be demonstrated.

What are the optimal components of an effective KoRV vaccine?

- The optimal immunogen must be defined through independent tests of various substrates.
- For FeLV vaccination, at least five approaches to antigen preparation have been developed including viral antigen shed into culture supernatant, inactivated whole virus, viral envelope proteins expressed from bacteria or from canarypox vector, and viral DNA as vaccine, introduced alone or with cytokines.
- The efficacy of commercial FeLV vaccines has been rigorously compared in peer-reviewed studies. Whole inactivated virus appears to be most effective with protection measured at >90%.
- A novel general approach for inactivation of retroviruses involves preferential chemical crosslinking of retroviral *gag* proteins, including the nucleocapsid protein, resulting in inactivation of infectivity with preservation of structurally and functionally intact envelope glycoproteins, which may have advantages as a whole inactivated vaccine immunogen. This approach may be suitable for development of a candidate KoRV vaccine.

What factors influence the timing and approach to delivery of a KoRV vaccine?

- Studies are required to determine the optimal timing of vaccine delivery based on the age of vaccination at which the animal is best protected. FeLV vaccination is typically delivered early in life (8–9 weeks of age) with a booster after one year.
- Older cats are significantly less susceptible to FeLV infection; thus, less frequent boosting is required as the animal ages. For KoRV infection, the possibility of age-related resistance has not been examined.
- The natural routes of KoRV exposure must be identified to optimize the approach to vaccine delivery. Considerations include evidence in support of endogenous virus, milk-borne virus, perhaps sexually-transmitted virus.
- The possibility of a post-infection KoRV vaccine should be examined.
- The possibility of a therapeutic KoRV vaccine should be examined, i.e., a vaccine to be delivered after KoRV-associated disease is evident.

What assays might be most useful to quantify protection after vaccination?

- Quantitative, reproducible assays are needed to define, verify and quantify the effectiveness of vaccination. Assays should measure viremia, proviral load and viral RNA after challenge.
- For FeLV, ELISA for p27Gag and quantitative real-time PCR are the most widely used assays for this purpose. KoRV-specific reagents could be developed to recapitulate these assays in the KoRV model.
- "Preventable fraction" (PF) is a key concept in evaluating vaccine effectiveness. As described for FeLV vaccines, the calculation of PF takes into account innate resistance to infection.

4 How might collaborative interactions best proceed toward treatment and prevention strategies for KoRV infection?

- Pathogenesis must be more completely understood with respect to the affected tissues in natural infection and the typical course(s) of disease, including potential differential pathogenesis and susceptibility to intervention of KoRV-A vs. KoRV-B.
- The complex genetics of the KoRV system, with possible spread from both exogenous and endogenous virus sources, confounds drug and vaccine development. Further analysis of wild and captive populations is needed to unravel this.
- To translate the success of other retroviral vaccines to KoRV, it will be important to determine whether natural immune clearance occurs in KoRV infection. Serology will be valuable to establish whether there are animals that were exposed naturally and cleared infection; this finding would indicate whether self-limiting infection occurs through an effective immune response.
- If self-limiting infection is identified, the immune correlates of protection should be determined in order to optimize the vaccine.
- The most relevant mode of transmission needs to be determined. If milk-borne transmission is key, for example, would it be possible to vaccinate the mother and transmit transient protection to the nursing joey?
- For the purpose of drug and vaccine evaluation in meaningful challenge models, it will be important to determine if KoRV-free koalas occur in captivity or in the wild.

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Population Management Strategies for Reducing Koala Retrovirus (KoRV) Impacts on Captive Populations

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Abstract. This manuscript summarizes the break-out session held on population management strategies for reducing koala retrovirus (KoRV) impacts on captive populations at the *Koala Conservation Workshop: The koala and its retroviruses: implications for sustainability and survival* held at San Diego Zoo, April 17–18, 2013. The goals of this break-out session were to identify research and population management activities that could facilitate reducing KoRV impacts on captive koala populations. Although both goals were met and suggested activities identified, no long term modifications to current breeding strategies were agreed upon due to current gaps in knowledge about KoRV. Herein, proposed research and population management activities developed at the workshop are described.

Ivy, Jamie A. 2014. Population management strategies for reducing koala retrovirus (KoRV) impacts on captive populations. In *The Koala and its Retroviruses: Implications for Sustainability and Survival*, ed. Geoffrey W. Pye, Rebecca N. Johnson and Alex D. Greenwood. *Technical Reports of the Australian Museum, Online* 24: 97–98.

Cooperative breeding programs sponsored by regional zoo associations typically utilize breeding strategies designed to retain gene diversity and limit inbreeding. These goals are accomplished by iteratively breeding individuals with the lowest average kinship (or relationship) within a population; since these animals have the fewest relatives, they are genetically underrepresented and have higher probabilities of possessing genetic variation at risk of being lost. Can current breeding strategies be modified to reduce koala retrovirus (KoRV) expression in captive populations, while still maintaining the genetic and demographic viability of those populations?

KoRV is known to be present in captive koalas throughout the US, Europe, and Australia. Given the regional representatives present at the Koala Conservation Workshop, the break-out session participants focused primarily on the management of populations in the US and Europe. Because additional koalas are expected to be imported into these populations from captive Australian populations in the next five years, ways in which future imports might impact the prevalence of KoRV in the US and Europe were considered alongside breeding strategy modifications.

The discussion on possibilities for reducing KoRV expression in captive populations of koalas was primarily focused around two broad topics. The first topic was the need for additional, collaborative research on KoRV. In particular, it was suggested that increased testing for KoRV is needed and institutions that hold large numbers of captive koalas in the US and Australia should collaborate on both prospective and retrospective research. Studies on the association between disease and KoRV status are greatly needed to better inform modifications to population management. The second topic of focus was the implication of multiple KoRV variants being present in captive koala populations. Both KoRV-A and KoRV-B are present in the captive US population, with KoRV-A being the predominant variant. Because many break-out session participants were particularly concerned about disease associated with KoRV-B, actions or strategies that would limit or eliminate this variant in captive populations in the US and Europe were of particular interest.

Proposed research activities

Short term (2–3 years)

- Testing for KoRV should be continued, particularly throughout captive and wild populations in Australia. Australian samples from an initial study are currently waiting testing in the US, with results expected in April 2013. Additional testing would better quantify the prevalence and distribution of the KoRV-B variant.
- The US has initiated a pilot study to investigate KoRV-related mortality in approximately 23 animals, but a larger test group should be identified. Further characterization of KoRV-related disease and mortality is needed to better quantify the risks to captive animals.
- Wildlife biologists working on koalas should be trained on proper biological sampling techniques, so that wild populations can be tested for KoRV. Increasing veterinarian involvement in field research would help generate additional KoRV-related data on wild populations.
- Research results should be widely disseminated to facilitate international involvement in both generating KoRV-related data and identifying actions and strategies that globally reduce KoRV expression in captive koalas.

Intermediate term (3–5 years)

- Research projects related to determining if there are management practices that may be contributing to KoRV-related disease in captive koalas should be initiated. For example, some factors that could be investigated include general husbandry, nutrition, harem size, and transfer of animals between institutions. Determining if any management practices contribute to KoRV-related disease could identify alternate methods, which might be unrelated to breeding strategies, for reducing KoRV expression in captive populations.

Long term (10+ years)

- Methods for better integrating ex-situ and in-situ research should be developed to improve global koala conservation and population viability.

Proposed population management activities

Short term (2–3 years)

- KoRV-A and KoRV-B koalas in the US should be managed as separate subpopulations. Temporarily managing the KoRV-B koalas as a separate subpopulation would allow additional data on the prevalence and health impacts of the variant to be collected, while limiting the spread of KoRV-B in the US population.

Intermediate term (3–5 years)

- Cooperative, global management of captive koala populations should be encouraged and facilitated by both regional zoo associations and institutions holding captive koalas. In order for population management strategies to be effective at reducing KoRV expression in captive populations, regions that exchange animals must collaborate to adopt similar management strategies.
- A business plan should be developed that would provide funds to support continued research and KoRV testing. If population management strategies are to be modified based on the KoRV status of individuals, all individuals participating in breeding programs must be tested.

Long term (10+ years)

- Gene diversity of captive koala populations in the US and Europe should be improved. The current levels of inbreeding in these populations suggest that increasing gene diversity is necessary for these populations to remain genetically viable over the long term.
- If KoRV-B continues to be of particular concern, the possibility of establishing a captive population that is KoRV-B negative should be considered. This population could then serve as a reservoir of animals that are free of the KoRV-B variant.

Conclusion

The goals of this break-out session were to identify research and population management activities that could facilitate reducing KoRV expression in captive koala populations. Although both goals were met and the preceding activities identified, no long term modifications to current breeding strategies were agreed upon due to the current gaps in knowledge about KoRV. Because many break-out session participants were particularly concerned about disease associated with KoRV-B, a proposed short term activity was to manage KoRV-A and KoRV-B koalas in the US as separate subpopulations. Managing the KoRV-B koalas as a separate subpopulation would limit the spread of the variant in the US population while additional data on the prevalence and health impacts of the variant are collected. However, a number of break-out session participants cautioned that managing two separate subpopulations of koalas in the US is not a sustainable option; there is not enough space for koalas in US zoos to manage two subpopulations that are of suitable sizes to be both genetically and demographically viable over the long term. In fact, the inability to maintain two subpopulations was previously demonstrated when a portion of the US population was separately managed due to concerns related to hip dysplasia. Although long term options for reducing KoRV expression in captive koala populations are yet to be identified, the proposed research is expected to significantly inform possible population management modifications.

Acknowledgments. Many participants in the Koala Conservation Workshop contributed to the discussion on activities to reduce the impacts of KoRV on captive populations. Thank you to everyone who participated and shared their ideas.

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Koala Retrovirus (KoRV): Are Humans at Risk of Infection?

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Abstract. This manuscript summarizes the break-out session held on koala retrovirus (KoRV): Any risks of human infection? at the *Koala Conservation Workshop: The koala and its retroviruses: implications for sustainability and survival* held at San Diego Zoo, April 17–18, 2013. The goals of this break-out session were to discuss the zoonotic risk of koala retroviruses, the necessity to test human populations for exposure, and precautions to be taken to protect humans who transport or handle koalas *(Phascolarctos cinereus)*. Currently there is no evidence to support the zoonotic potential of KoRV, and the necessity to test humans for KoRV infection needs to be further justified. We recommend strict compliance with standard precautions when handling animals.

Xu, Wenqin, and Jonathan P. Stoye. 2014. Koala retrovirus (KoRV): are humans at risk of infection? In *The Koala and its Retroviruses: Implications for Sustainability and Survival*, ed. Geoffrey W. Pye, Rebecca N. Johnson and Alex D. Greenwood. *Technical Reports of the Australian Museum, Online* 24: 99–101.

Zoonotic potential of retroviruses

Based on their genomic structure, retroviruses are generally classified as either simple or complex. A number of complex retroviruses from nonhuman primates such as simian immunodeficiency virus, simian leukemic virus, and foamy virus have the capacity to jump species and infect humans. Koala retrovirus (KoRV), by contrast, is a simple retrovirus of the gammaretrovirus genus, whose members do not contain the accessory proteins required to counteract human cell restriction factors. Replication of a gammaretrovirus in human cells is therefore largely inhibited by human restriction factors, such as APOBEC 3 enzymes and tripartite motif (TRIM) proteins.

Viruses made in non-primate cells will also be inactivated by the complement system following binding of naturally occurring antibodies to the alpha gal epitope.

In the extensive studies and discussions that followed the discovery of a putative human retrovirus, xenotropic murine leukemia virus-related virus (XMRV), which was first isolated from human prostate cancer tissues but later shown to be a laboratory contaminant, the likelihood of a gammaretrovirus jumping species and replicating efficiently in humans was proposed and dismissed. Some gammaretroviruses have been shown to have the ability to infect human cells efficiently in culture yet show no evidence of transmission to humans, for example, feline leukemia virus (specifically FeLV subgroup B) and porcine endogenous retrovirus (PERV), both of which are related to KoRV. While 2–3% of U.S. domestic cats are infected with FeLV, which causes cat leukemia and lymphoma, FeLV infection of humans has not been detected (Levy *et al*., 2008; Hartmann, 2012). Neither has PERV been demonstrated to be zoonotic, PERV has not been detected in patients who

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have received clinical xenotransplantation of pig materials. In fact, when immunosuppressed small animals or nonhuman primates are inoculated with PERV, no infection occurs.

Transmission of KoRV

The gibbon ape leukemia virus (GALV), a highly oncogenic gammaretrovirus capable of inducing myeloid leukemia in juvenile gibbons, is conspecific with KoRV. Infectious GALV viruses have been detected in the blood, urine, and feces of infected gibbons and are known to be transmitted *in utero*, postnatally, or through contact of virus-free gibbons with infected gibbons. The koala retrovirus KoRV-A (subtype A) is an endogenous infectious retrovirus and transferred both horizontally and vertically in the germline, while the newly discovered KoRV-B (subtype B) appears to be exogenous (Xu *et al.,* 2013). KoRV-B is presumably transmitted either *in utero* or through the dam's milk, as evidenced by the detection KoRV-B from eight offspring of KoRV-B positive dams including two joeys ejected from the pouch of KoRV-B positive dams while the offspring of KoRV-B positive sires and KoRV-B negative dams are KoRV-B negative. Whether KoRV-B can be transmitted through contact of uninfected koalas *(Phascolarctos cinereus)* with infected koalas is as yet unknown. KoRV has been detected in blood and feces, but the assessment of virus in saliva and urine has not yet been performed.

Discussion

What we currently know about the risk of human infection by KoRV

The zoonotic potential of KoRV is generally compared to that of FeLV, a related virus that infects domestic cats, which has not been found to infect caregivers or to be associated with any disease in humans. To date, there is no evidence that any gammaretrovirus can jump species to infect humans, and both KoRV-A and KoRV-B are associated with diseases in koalas only. Establishing actual zoonotic risk from KoRV, however, requires more data on:

- *Routes of transmission:* whether KoRV-B can be transmitted from infected adult koala to uninfected adult koala through contact.
- *Mutation rates:* whether KoRV-A or B can mutate at rates high enough to circumvent innate human immune and antiretroviral response.
- *Epizoonotic transmission:* whether KoRV can be transmitted to predators of koalas.
- *Pathogenic potential:* The precise role of KoRV-A and B in koala disease remains to be firmly established.
- *Animal models for KoRV:* whether KoRV-A and B can replicate in non-human primates and cause leukemia or lymphoma.
- *Replication of virus in humans*: whether KoRV can replicate efficiently in primary human cells.
- *Mechanisms of human KoRV inhibition:* how different human restriction factors inhibit KoRV replication.

Testing humans for KoRV infection

Although the evidence currently suggests no zoonotic potential for KoRV, including exogenous KoRV-B, prudence suggests the need to exclude even the remote possibility of KoRV infection by testing the small population that comes into close contact with koalas and animal viruses (animal keepers, veterinarians, registered veterinary technicians, researchers, and possibly tourists with koala contact). PCR assays to detect KoRV specific sequences in blood and tissue samples are available, and ELISA and western blot assays can also be developed to test for KoRV antibody levels and viral protein expression. Any program to test human samples for KoRV infection, however, will depend on the following considerations:

- The justifiability of using resources to test humans for KoRV when there is currently no evidence of zoonotic transmission.
- How to interpret any positive results, given that laboratory contamination (as with XMRV), the sensitivity of modern assays, and cross-reactions can all result in false positives.
- Particularly in the absence of any documented transmission events even a low frequency of false positives is potentially controversial.

Guidelines for animal caregivers

Since zoonotic diseases can threaten the health of animal caregivers, zoos generally institute rigorous precautions, especially for the handling of non-human primates and other species at high risk for the transmission of serious zoonoses. Guidelines for handling nonhuman primates are available to prevent highly infectious zoonotic diseases.

Until now, koalas have not been considered in this category and are therefore handled using standard hygiene procedures. However, with the discovery of exogenous retrovirus KoRV-B, new questions have arisen about the potential risk to humans. While it is not yet clear whether KoRV-B presents a zoonotic threat to humans, contact with animals always carries some risk of disease transmission. At present there are no specific guidelines for handling koalas. To reduce the possibility of zoonotic diseases, we recommend strict adherence to standard precautions when handling animals especially when performing necropsies, and that every new koala should be presumed as KoRV-B positive before tests.

To decide whether it is necessary to establish more stringent guidelines for handling koalas, researchers will need to collect more data on:

- Viral load in koala urine, feces, and saliva.
- Effect of human complement on koala-derived viruses.
- Replication of KoRV in primary human cells.
- Inhibitory effects of different human restriction factors on the replication of KoRV.
- Reservoir for KoRV in animals.

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Koala Retrovirus Workshop Conclusion. The Future of KoRV Research—Foundational and Applied

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Abstract. This manuscript summarizes the conclusion session of the *Koala Conservation Workshop: The koala and its retroviruses: implications for sustainability and survival* held at San Diego Zoo, April 17–18, 2013. The main goals of the workshop were to determine the current state of foundational research of koala retrovirus (KoRV), the future foundational research needed, to initiate the need for applied research, and to create a collaborative international effort on KoRV that would directly help the sustainability and survival of both captive and free-ranging koalas *(Phascolarctos cinereus)*. The seven areas of future collaborative research of the workshop were determined to be: *(1)* Does KoRV cause disease in koalas? *(2)* Does KoRV cause population declines? *(3)* Are there KoRV-free koalas? *(4)* What is the importance of the variants of KoRV? *(5)* Is KoRV or its variants horizontally transmitted? *(6)* Do koalas develop an immune response to KoRV? *(7)* What is the role of prevention and therapy in free-ranging and captive koalas?

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Does KoRV cause disease in koalas?

There is plenty of supportive evidence to suggest that koala retrovirus (KoRV) causes lymphoid neoplasia in koalas *(Phascolarctos cinereus)* (Canfield *et al*., 1987, Canfield *et al*., 1988; Worley *et al*. 1993, Hanger *et al.,* 2000; Tarlinton *et al*., 2005), but, at this time, no-one has definitive proof of this. A missing resource impeding progress is the lack of an annotated koala genome. However, as presented by Rebecca Johnson, this situation is changing rapidly with the sequencing of a koala genome and transcriptome which is now in the annotation stage (Johnson *et al*., 2014, this volume). Important evidence for a causal role in disease by KoRV that is currently lacking is integration site differences of KoRV in diseased versus healthy tissues. It was agreed that this is crucial information that should be determined as soon as possible. It has been suggested that KoRV may cause disease by immunosuppression (Fiebig et al., 2006; Denner, 2014, this volume). However, KoRV positive koalas mount a strong immune response to antigens derived from *Chlamydia* (Timms, 2014, this volume). The consequence of KoRV on immune response thus requires further investigation to determine if KoRV has a broad, specific, or no effect on koala immune function. It was identified that there was a need to standardize both the collection of tissue samples from suspected KoRV-related diseased koalas and the epidemiological survey methods used to examine the data. In addition, studies looking at the potential immune suppressive effects of KoRV were identified as an important need.

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Are there KoRV-free koalas?

The studies by Tarlinton *et al.*, 2006 and Simmons *et al.*, 2012 suggest that potential KoRV-free free-ranging populations may be diminishing. It was identified that that the definition of KoRV-free koalas should be based on genomic information that shows that the individual koala is free of the potential to give rise to the virus. This will require an improvement in testing sensitivity from currently used methods. More sampling surveys are needed, particularly in southern Australia. If KoRV-free koalas are identified, then isolation should be considered, if sufficient numbers exist for a sustainable population.

Does KoRV cause population decline?

While KoRV likely has a significant effect on captive populations (Gillett, 2014, this volume; Hanger *et al.*, 2000, Miyazawa, 2014, this volume; Mulot, 2014, this volume; Pye *et al.*, 2014, this volume), there is little data on the prevalence of KoRV-associated disease in free-ranging populations (Hanger & Loader, 2014, this volume). Anecdotally there appears to be quite a difference in prevalence of expression of KoRV-associated disease across Australia, particularly between northern and southern areas. Even in Queensland where there is 100% prevalence of KoRV-A (Tarlinton *et al.*, 2006; Simmons *et al.*, 2012), there appears to a great deal of variation in the apparent prevalence of KoRV-associated disease (e.g., south-eastern Queensland versus central Queensland; Drs Amber Gillett and Sean FitzGibbon, pers. comm. 2013).

Much more data needs to be collected using standardized methods across the whole range of koalas. Current data on koala population declines is generally incomplete in Australia and typically does not include disease status. A considerable collaborative effort is needed to ensure sufficient standardized data from across the range to make a study of population or species decline meaningful as opposed to anecdotal. Comparison with data from a KoRV-free populations would be ideal if such populations truly exist.

What is the importance of the variants of KoRV?

With the recognition and recent sequencing of variants of KoRV, a standardized system is required to classify known and future variants. The system could be based on biological assay to determine the functional significance of identified variation, molecular clone development for comparative analysis and potential vaccine and serological resource development, serology where possible to determine prevalence in both captive and wild populations rapidly, samples from diverse populations to examine both epidemiology and among variant variation, uniform standardization and definitions to prevent nomenclature confusion in the literature, and through the sharing of reagents. Efforts should be made to determine how the viruses were generated e.g., by point mutation or recombination.

Currently the significance of KoRV variants on disease expression is unknown. It has been suggested that KoRV-B may be more pathogenic (Xu *et al.*, 2013). Examination of correlation of variant presence and mortality data over large populations in Australia may help determine the significance of the individual variants of KoRV. Until this is done, caution should be taken with mixing koalas of known differing variant status (e.g., KoRV-B positive koalas with KoRV-B negative koalas).

Is KoRV or its variants horizontally transmitted?

While it is well understood that endogenous KoRV is passed from koala to koala via the germ line, it is not known how exogenous forms of KoRV are transmitted or whether any KoRVs are currently being transmitted horizontally. It is possible that exogenous KoRV may be transmitted during copulation, in utero, via milk, via pap (the soft feces from the mother that the joey feeds on prior to beginning to eat *Eucalyptus*), or by direct contact (e.g., males fighting). Findings of KoRV-B positive tissue from 3-month-old joeys are suggestive that either in utero or via milk transmission occurs (Maribeth Eiden, pers. comm. 2013).

Opportunities may exist in the captive situations in Australia, Japan, and North America where KoRV-B positive and KoRV-B negative koalas have comingled to look at prevalence, integration- site distributions, pedigrees, and social interactions in order to model possible routes of transmission. In addition, longitudinal studies on populations where exogenous transmission appears to be occurring (e.g., Kangaroo Island) may be helpful in determining routes of horizontal transmission.

Do koalas develop an immune response to KoRV?

We need to determine if KoRV-infected koalas are tolerant to the virus, is there evidence of clearing of natural infection, are there resistant populations, and are the perceived cycles of infection real. Further development and standardization of assays in koalas is an important part of the process.

What is the role of prevention and therapy in free-ranging and captive koalas?

There are a large number of antiretroviral drug therapies available for HIV+ humans. Many of these drugs act to prevent replication of the virus and therefore prevent repeated cycles of infection. In koalas, we don't know whether cycles of infection occur or if they do, their role in the expression of clinical disease. Consequently we don't know if treatment would be beneficial in koalas infected with endogenous KoRV-A. Prophylactic treatment of female koalas negative for exogenous variants of KoRV (KoRV-B) may prove beneficial at times of breeding (e.g., if KoRV-B positive washed semen is used for artificial insemination).

Vaccination holds more promise due to the success of developing vaccines for FeLV. Uniform standards and definitions for challenge and protection will be required.

Progress in these seven areas will hopefully enhance our understanding of KoRV biology and lead to practical applications that can benefit captive and free-ranging koala health.

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